PATENT
Attorney Docket No. 10070485-02-US
Customer No. 27,495

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent Application of

Applicant: Michael BORNS

Application No.: 10/805,650

Filing Date: 19 March 2004

Group Art Unit: 1637

Confirmation No.: 9645

Examiner: M. STAPLES

Title: DNA POLYMERASE FUSIONS AND USES THEREOF

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

APPEAL BRIEF UNDER BOARD RULE 37 C.F.R. § 41.37

In support of the Notice of Appeal filed 6 February 2009, and pursuant to Board Rule 41.37, Appellant presents this brief and pays the fee of $270.00 (small entity) required under 37 C.F.R. § 41.20(b)(2). This Appeal Brief is due by 6 April 2009 and is timely filed.

This Appeal responds to the final rejection of claims 1-10, 13, 15, 25-29, and 40-52 set forth in the Final Office Action mailed 8 October 2008.

If any additional fees are required or if the submitted payment is insufficient, Appellant requests that the required fees be charged to Deposit Account No. 50-3740.

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I. Real Party in Interest

Stratagene California, which has been renamed Agilent Technologies Research Corporation, is the real party in interest, as shown by the assignment recorded on 27 July 2004 at reel 015604, frame 0300. Agilent Technologies Research Corporation is a subsidiary of Agilent Technologies, Inc.

II. Related Appeals and Interferences

There are currently no other appeals or interferences, of which Appellant, Appellant's legal representative, or Assignee is aware, that will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. Status of the Claims

Claims 1-10, 12-18, 21-29, and 40-52 are pending in this application and are set forth in Appendix I. Claims 11, 19, 20, and 30-39 have been cancelled. Claims 12, 14, 16-18, and 21-24 are withdrawn from consideration. Claims 1-10, 13, 15, 25-29, and 40-52 stand finally rejected by the Examiner as noted in the Advisory Action mailed 23 December 2008. Appellant appeals the final rejection of claims 1-10, 13, 15, 25-29, and 40-52.

IV. Status of Amendments

Appellant's Amendment After Final filed 8 December 2008 has been entered. *See* Advisory Action mailed 23 December 2008. No subsequent amendments have been filed.

V. Summary of the Claimed Subject Matter

Claims 1-10, 13, 15, 25-29, and 40-52 are directed to methods of using a DNA polymerase fusion under conditions of high pH, where the DNA polymerase fusion comprises wild type *Pyrococcus furiosus* polymerase I fused to *Sulfolobus solfataricus* SSso7d protein and functions as a DNA polymerase. The conditions of high pH are defined in the claims as 9.3 to 12 (claims 1-4, 7-10, 13, 15, 25-29, 40, and 41) or 9.5 to 12 (claims 42-46). Claims 1, 3, 5, 7, and 9 are all independent claims and differ from each other with respect to the method of using the recited DNA polymerase fusion.

A. Claim 1 (DNA Synthesis at High pH)

Claim 1 is directed to a method for DNA synthesis at high pH and comprises contacting the Pfu-Sso7d DNA polymerase fusion with a nucleic acid template at pH 9.3-12.

Support for claim 1 can be found throughout the specification, including, for example, at page 5, lines 12-14; page 12, lines 17-20; page 29, lines 4-8; Fig. 2; page 59, lines 16-21; page 63, lines 14-24; and pages 78-82 (Example 3).

B. Claim 3 (Cloning of a DNA Synthesis Product at High pH)

Claim 3 is directed to a method for cloning of a DNA synthesis product at high pH and comprises:

a) providing a Pfu-Sso7d DNA polymerase fusion;

b) contacting the Pfu-Sso7d DNA polymerase fusion with a nucleic acid template at pH 9.3-12 so as to effect the template dependent synthesis of a DNA synthesis product; and

c) inserting the synthesized DNA product into a cloning vector.

Support for claim 3 can be found throughout the specification, including, for example, at page 5, lines 15-18; page 12, lines 17-20; page 29, lines 4-8; Fig. 2; page 59, lines 16-21; page 63, lines 14-24; and pages 78-82 (Example 3).

C. Claim 5 (Sequencing DNA at High pH)

Claim 5 is directed to a method for sequencing DNA at high pH and comprises:

(a) contacting a template DNA strand with a sequencing DNA primer;

(b) contacting the DNA of step (a) with a Pfu-Sso7d DNA polymerase, deoxyribonucleoside triphosphates, and a chain-terminating nucleotide analog;

(c) incubating the mixture of step (b) under conditions sufficient to synthesize at pH 9.3-12 a random population of DNA molecules complementary to the DNA molecule, wherein the synthesized DNA molecules are shorter in length than the first DNA molecule and wherein the synthesized DNA molecules comprise a terminator nucleotide at their 5' termini; and

(d) separating said synthesized DNA molecules by size so that at least a part of the nucleotide sequence of said first DNA molecule can be determined.

Support for claim 5 can be found throughout the specification, including, for example, at page 5, lines 19-28; page 12, lines 17-20; page 29, lines 4-8; Fig. 2; page 59, lines 16-21; page 63, lines 14-24; and pages 78-82 (Example 3).

D. Claim 7 (Linear or Exponential PCR Amplification at High pH)

Claim 7 is directed to a method of linear or exponential PCR amplification at high pH for site-directed or random mutagenesis comprising the step of incubating a reaction mixture comprising a nucleic acid template, at least one PCR primers, and a Pfu-Sso7d DNA polymerase fusion under conditions which permit amplification of said nucleic acid template at pH 9.3-12 by the Pfu-Sso7d DNA polymerase to produce a mutated amplified product.

Support for claim 7 can be found throughout the specification, including, for example, at page 6, lines 1-5; page 12, lines 17-20; page 29, lines 4-8; Fig. 2; page 59, lines 16-21; page 63, lines 14-24; and pages 78-82 (Example 3).

E. Claim 9 (Reverse Transcriptase at High pH))

Claim 9 is directed to a method of reverse transcriptase PCR at high pH comprising the step of incubating a reaction mixture comprising a nucleic acid template, at least one PCR primer, and a Pfu-Sso7d DNA polymerase fusion under reaction conditions which permit amplification of said nucleic acid template at pH 9.3-12 by the Pfu-Sso7d DNA polymerase fusion to produce an amplified product.

Support for claim 9 can be found throughout the specification, including, for example, at page 6, lines 6-9; page 12, lines 17-20; page 29, lines 4-8; Fig. 2; page 59, lines 16-21; page 63, lines 14-24; and pages 78-82 (Example 3).

Claims 2, 4, 6, 8, 10, 13, 15, 25-29, and 40-52 depend directly or indirectly from independent claims 1, 3, 5, 7, and 9.

VI. Grounds of Rejection

A. Claims 1-4, 7-11, 13, 15, 19, 25-30, and 40-48[1] stand rejected under 35 U.S.C. § 103(a) as allegedly obvious over WO 01/082501 (*Wang*).

B. Claims 5 and 6 stand rejected under 35 U.S.C. § 103(a) as allegedly obvious over *Wang* in combination with *Sanger*.

C. Claims 49-52 stand rejected under 35 U.S.C. § 103(a) as allegedly obvious over *Wang* in view of *Dietrich*.

[1] Appellant notes that claims 11, 19, and 30 have been cancelled.

VII. Argument

A. *Wang* Does Not Render Obvious Claims 1-4, 7-10, 13, 15, 25-30, and 40-48

1. Claims 1-4, 7-10, 13, 15, 25-30, and 40-41

The Examiner rejects claims 1-4, 7-11, 13, 15, 19, 25-30, and 40-41[2] under 35 U.S.C. § 103(a) as allegedly obvious over WO 01/082501 (*Wang*). Final Office Action at page 4. Appellant respectfully requests reversal of this rejection of claims 1-4, 7-10, 13, 15, 25-29, and 40-41.

To establish a *prima facie* case of obviousness, the prior art reference (or references when combined) must teach or suggest all the claim limitations. *See* M.P.E.P. § 2143. Appellant submits that *Wang*, alone, or in combination with the state of the art, does not teach all of the elements of the rejected claims.

Wang does not teach or suggest the pH range of 9.3 to 12 recited in claims 1-4, 7-10, 13, 15, 25-29, 40, and 41. *Wang* teaches using a Pfu-Sso7d polymerase fusion with the standard reaction buffer for wild type Pfu polymerase, which contains 20 mM Tris-HCl (pH 8.8) as a buffering component. *Wang*, page 39, lines 13-16. In addition, *Wang* teaches a buffer containing Tris HCl with a pH of 9.0 for the DyNAzyme EXT, which is a mixture of DyNAzyme II DNA polymerase and a proofreading enzyme. Final Office Action at page 4; *Wang* at page 40, lines 13-15. Thus, as acknowledged by the Examiner, "Wang does teach at least pH 8.8 for the Pfu-Sso7d fusion polymerase and for the Taq polymerase (see 2nd sentence on p. 42) and teaches pH 9.0 for polymerases of DNAZYME EXT." Final Office Action at page 4. The Examiner argues that it would have been obvious as a matter of routine optimization to

2 Appellant notes that claims 11, 19, and 30 have been cancelled. Claims 2, 4, 6, 8, 10, 13, 15, 25-29, and 40-41 depend directly or indirectly from independent claims 1, 3, 5, 7, and 9.

increase the pH used with the Pfu-Sso7d fusion protein of *Wang* to 9.3 or 9.5. Office Action mailed 4 March 2008 at pages 5-6. For the reasons of record, Appellant asserts that it would not have been obvious to use the Pfu-Sso7d polymerase fusion of *Wang* in the claimed methods at a pH of 9.3 to 12.

The Federal Circuit has held that "a prima facie case of obviousness exists when the claimed range and the prior art range do not overlap but are close enough such that one skilled in the art would have expected them to have the same properties." *In re Peterson*, 315 F.3d 1325, 1329 (Fed. Cir. 2003). As noted in the general rule, an exception exists when the difference between the prior art and the claimed range are such that one of skill in the art would not have expected them to have the same properties. That exception applies here. As established through the Borns Declaration[3], one skilled in the art would *not* have expected that the reaction buffer in *Wang* would have the same properties if the pH were raised to 9.3 or 9.5. Specifically, one of skill in the art would have expected that increasing the pH of the reaction buffer disclosed in *Wang* to a pH of 9.3 would significantly impair the conditions for amplification with either the wild type Pfu DNA polymerase or the claimed Pfu-Sso7d fusion polymerase. Declaration at ¶ 16.

More specifically, Attachment B to the Declaration shows that although wild type Pfu DNA polymerase has optimal activity at pH 8.8, it loses this activity above pH 9, as acknowledged by the Office. Office Action mailed 4 March 2008 at page 3 ("[T]he declaration: (1) provides evidence only showing that a non-chimeric polymerase which is PFU Turbo loses activity above pH 8.8 (see Attachment B) . . . ."). The Declaration explains that *for the same*

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[3] *See* Attachment 1 to response filed 17 December 2007.

reasons, one of skill in the art would expect that a Pfu-Sso7d fusion polymerase containing a wild type Pfu DNA polymerase, such as the one disclosed in *Wang*, would similarly lose activity above pH 9. *See* Declaration at ¶ 16. The Borns Declaration stands unrebutted.

Thus, one of skill in the art would not be motivated to increase the pH of the reaction buffer (pH 8.8) disclosed in Example 6.1 of *Wang* to a pH of 9.3 because he would expect that such a change would significantly impair the conditions for amplification with either the wild type Pfu DNA polymerase *or the Pfu-Sso7d fusion polymerase*. *See* Declaration at ¶ 16. Accordingly, contrary to the Examiner's assertions, and absent the teachings of the present specification, one of ordinary skill in the art having experience working with DNA polymerases would expect the differences between the conventional pH used for Pfu-based PCR in *Wang* (about 8.3 to 8.8) and the pH recited in claims 1-4, 7-10, 13, 15, 25-29, 40, and 41 (9.3-12) to significantly impair the efficiency of PCR performance. *See* Declaration at ¶ 16.

In response, the Examiner cites Dietrich et al. (2002) ("*Dietrich*") as establishing that "it was known in the art that polymerases function at pH 9.5 and up to pH 10." Final Office Action at page 4. The Examiner further asserts that *Dietrich* "also support, as given below, that polymerase activity can be found over a broad pH range of pH 7 to pH 10 which is consistent with the teachings of Wang." *Id.* Thus, the Examiner concludes that "Applicant's opinion that one of ordinary skill in the art would not have expected the polymerase fusion of *Wang* to function at pH 9.5 is not supported by the teachings of the prior art." *Id.* at 4-5.

Appellant's response is two-fold. First and foremost, *Dietrich* is directed to a polymerase from *Pyroccocus abyssi* ("Pab"), whereas the claimed fusion protein comprises a *Pyrococcus furiosus* ("Pfu") DNA polymerase. It is improper for the Office to draw generalizations about DNA polymerases based on the biochemical properties of a single enzyme. This is particularly

true here because Appellant has shown in the Borns Declaration that the Pfu DNA polymerase loses activity above pH 9. This is not disputed. Moreover, it should not be disputed that it is the biochemical properties of Pfu DNA polymerase—not the Pab DNA polymerase—that are relevant to the presently claimed invention and the question of whether one of skill in the art would have been motivated to increase the pH of the Pfu-Sso7d polymerase fusion protein buffer to 9.3. The Examiner has not responded to this argument.

Secondly, there are no data supporting the statements in *Dietrich* that

> Pab was more active at pH 9 in glycine-NaOH buffer and pH 9.5 in CAPS buffer. Using Tris-HCl buffer, Pab retained more than 80% of its optimal activity between pH range 7-10 and appeared to possess an extended range of DNA polymerizing activity.

Dietrich at 92, first column. Instead, the only pH-dependency data reveal that the Pab DNA polymerase dramatically loses activity above the optimal pH of 9.0. Specifically, a review of the reference[4] allegedly supporting the statements in *Dietrich* about pH dependency demonstrates that while the Pab DNA polymerase ("Pol I") has an optimum pH of 8.5-9.0, the enzyme exhibits a marked decrease in activity when the pH rises above 9.0 and essentially loses activity altogether at pH 9.5. *Gueguen* at p. 5961 (Abstract) and p. 5965 (Fig. 4A). This is also consistent with the properties of the Pfu DNA polymerase, which has optimal activity at pH 8.8 but loses activity above pH 9. *See* Declaration at ¶ 13.

In response to this second argument, the Examiner asserts that *Gueguen* "teach that certain polymerases do not maintain significant activity at pH 9-10. However the instant claims do not recite the limitation of 'significant activity' and thus the teachings of Gueguen make

[4] Gueguen *et al.*, Eur. J. Biochem. 268, 5961-69 (2001) ("*Gueguen*"); *see* Attachment A to Amendment After Final Rejection Under 37 C.F.R. § 1.116 filed 8 December 2008.

obvious the currently claimed invention, as Gueguen necessarily teach that polymerase have some activity at pH 9-10." Advisory Action mailed 28 December 2008 at page 2. Appellant's response is that *Gueguen*, like *Dietrich*, refers to Pab DNA polymerase, not Pfu DNA polymerase. Therefore, the evidence about Pab DNA polymerase does not refute the evidence of record showing that the Pfu DNA polymerase loses activity above pH 9. *See* Declaration at ¶ 13. Second, *Gueguen* shows that while Pab Pol I has optimal activity at pH 9.0, it retains only about 20% of its activity at a pH between 9 and 9.5 and has essentially no activity at pH 9.5, confirming that one of skill in the art would understand that slight increases above the optimal pH can have a significant effect on DNA polymerase activity. *Gueguen* at p. 5961 (Abstract) and p. 5965 (Fig. 4A). Thus, even assuming that the Borns Declaration was not in evidence, the pH dependency data of Pab Pol I DNA polymerase in *Gueguen* would not motivate one of skill in the art to raise the pH to 9.3-12 when using a Pfu DNA polymerase or a fusion protein comprising the same. In fact, in view of the data in *Gueguen*, one of skill in the art would be compelled not to use the Pab DNA polymerase, or another DNA polymerase with a similar pH profile, at a pH above 9 because doing so would essentially destroy about 80% or more of the enzyme's activity. Rather, one of skill in the art would be motivated to use the Pab Pol I DNA polymerase at its optimal pH (8.5-9.0). *Gueguen* at p. 5961 (Abstract) and p. 5965 (Fig. 4A).

The Examiner also argues that "[f]urther as Dietrich teach after Gueguen, Dietrich is disclosing the new teaching of some polymerases functioning [*sic*, functioning] well at the claimed pH range." Advisory Action mailed 28 December 2008 at page 2. Appellant has addressed the teachings of *Dietrich* with respect to the Pab DNA polymerase above. If the Examiner is referring to polymerases other than the Pab DNA polymerase, Appellant requests

that the Examiner identify which polymerases allegedly function "well at the claimed pH range" and explain the purported relevance of any such polymerases to the claimed methods.

Another exception to the general rule that "'the discovery of an optimum value of a variable in a known process is normally obvious,' . . . is where the parameter optimized was not recognized in the prior art as one that would affect the results." *Ex parte Whalen II*, No. 2007-4423, 2008 WL 2957928, *8 (B.P.A.I. July 23, 2008)[5] (citations omitted). In *Whalen II*, the Board found that "the Examiner has not pointed to any teaching in the cited references, or provided any explanation based on scientific reasoning, that would support the conclusion that those skilled in the art would have considered it obvious to 'optimize' the prior art compositions by increasing their viscosity to the level recited in the claims." *Id.* In fact, the evidence of record in *Whalen II* established that low viscosity was a desired property of the prior art compositions. *Id.*

Similarly, in this application, the Examiner has not provided any evidence or any explanation based on scientific reasoning that one of skill in the art would have considered it obvious to optimize the Pfu-Sso7d fusion polymerase compositions of *Wang* by increasing the pH above 9, and in particular to the claimed range of 9.3 to 12. Rather, the evidence of record establishes that a pH below 9 was a desired property for compositions comprising Pfu or a Pfu-Sso7d fusion polymerase. In fact, the skilled artisan would have expected that raising the pH of the reaction buffer in *Wang* to 9.3 or higher would impair—not enhance—the efficiency of the disclosed Pfu-Sso7d fusion polymerase. Declaration at ¶¶ 16-18.

Also, it appears that in Example 6.1, *Wang* used a commercially available reaction buffer

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[5] A copy of this precedential opinion was submitted as Attachment B to the Amendment After Final Rejection Under 37 C.F.R. § 1.116 filed 8 December 2008.

for Pfu. Declaration at ¶ 15. One of ordinary skill in the art would not have been motivated to increase the pH of this reaction buffer as a matter of routine optimization for the additional reason that, as a commercial product, the reaction buffer would have already been optimized. *See* Declaration at ¶ 16. As discussed above, the Examiner's citation of *Dietrich* for the general principle that polymerases function at pH 9.5 and up to pH 10 is misplaced and does not address the evidence of record showing that it was known in the art that the Pfu polymerase loses its activity above pH 9. Thus, the art teaches away from increasing the pH buffer of *Wang* above 9 based on the expectation and understanding that doing so would impair the efficiency of *Wang's* Pfu-based fusion protein. *Whalen II*, 2008 WL 2957928, at *9 ("when the prior art teaches away from the claimed solution as presented here . . ., obviousness cannot be proven merely by showing that a known composition could have been modified by routine experimentation or solely on the expectation of success; it must be shown that those of ordinary skill in the art would have had some apparent reason to modify the known composition in a way that would result in the claimed composition.").

The Examiner attempts to distinguish *Whalen II*, asserting that

> the decision in Ex Parte Thomas J. [*sic*, Whalen II] relies on the fact that the claimed invention had structural differences from the previously patented invention, which were not found in the previous specification of that patent, and which then imparted different and non-obvious properties to the claimed composition. Thus the claimed composition was allowable over the previously patented composition. However there is no support in the instant specification for the claimed fusion polymerase to have any structural difference from the fusion polymerase disclosed by Wang. In effect Applicant is arguing that a newly discovered property inherent to an already disclosed fusion polymerase is patentable. Discovery of a new property inherently present in the prior art does not necessarily make the claimed invention patentable (see MPEP § 2112 I.)

Advisory Action mailed 23 December 2008, at page 2.

Appellant's response is that claims 1-4, 7-10, 13, 15, 25-29, 40, and 41 are not directed to a DNA polymerase fusion but rather to methods of using a DNA polymerase fusion at a pH of 9.3-12. And the methods of using the DNA polymerase fusion at a high pH, as recited in the claims, was neither taught nor suggested by *Wang*.

"In determining the differences between the prior art and the claims, the question under 35 U.S.C. 103 is not whether the differences <u>themselves</u> would have been obvious, but whether the claimed invention <u>as a whole</u> would have been obvious. *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 218 USPQ 871 (Fed. Cir. 1983); *Schenck v. Nortron Corp.*, 713 F.2d 782, 218 USPQ 698 (Fed. Cir. 1983) . . . ." M.P.E.P. § 2141.02. Before Appellant's discovery, there was a perceived need in the art to use a pH buffer below pH 9 when using a Pfu polymerase (either as a non-fusion or fusion polypeptide). Appellant, on the other hand, unexpectedly found that raising the pH above 9 actually enhances rather than impairs the PCR performance efficiency of a Pfu fusion polymerase. Because that insight was contrary to the expectations and understandings of the art, it would not have been obvious to modify the teachings of *Wang* to arrive at the methods of the presently claimed invention reciting a high pH of 9.3 to 12. *See Schenck*, 713 F.2d at 785, 218 USPQ at 700 ("Because that insight was contrary to the understandings and expectations of the art, the structure effectuating it would not have been obvious to those skilled in the art."). Accordingly, for the reasons of record, the Borns Declaration provides persuasive evidence showing that it would not be *prima facie* obvious to "optimize" the buffer used in *Wang* by increasing the pH to 9.3. In fact, the art teaches away from doing just that.

Accordingly, Appellant submits that *Wang* fails to teach or suggest all elements of claims 1-4, 7-10, 13, 15, 25-29, and 40-41 and, thus, does not render those claims obvious. For at least

this reason, Appellant requests that the Board reverse the rejection of these claims as unpatentable under 35 U.S.C. § 103 over *Wang*.

2. Claims 42-46

Claims 42-46 depend from independent claims 1, 3, 5, 7, and 9 and recite that a pH of 9.5 to 12 rather than 9.3 to 12, as recited in the independent claims. For the same reasons that *Wang* fails to teach or suggest a pH from 9.3-12, as discussed above with respect to claims 1-4, 7-10, 13, 15, 25-29, 40, and 41, Appellant submits that *Wang* also fails to teach or suggest a pH from 9.5-12. Accordingly, Appellant requests that the Board reverse the Examiner's rejection of claims 42-46 as unpatentable under 35 U.S.C. § 103 over *Wang*.

3. Claims 47 and 48

The Examiner rejects claims 47 and 48[6] under 35 U.S.C. § 103(a) as allegedly obvious over *Wang*. Final Office Action at page 5. Appellant respectfully requests reversal of this rejection for the reasons of record. Namely, *Wang* fails to teach or suggest a pH from 9.5 to 12, as recited in claims 47 and 48.

Claim 47 depends from claim 42 and recites that the DNA polymerase fusion is part of a blend comprising a second DNA polymerase. Claim 48 depends from claim 47 and recites that the second DNA polymerase is *Pfu*.

Appellant requests reversal of the rejection of claims 47 and 48 for the additional reason that *Wang* fails to teach or suggest a blend comprising a polymerase fusion and a second DNA polymerase, let alone a blend where the second DNA polymerase is a *Pfu* DNA polymerase. Although *Wang* teaches mixtures of non-fusion polymerases, it also teaches that those mixtures

[6] Although the rejection lists claims 49-52, only claims 47 and 48 are discussed. Thus, Appellant understands that this rejection pertains to claims 47 and 48—not claims 49-52.

require one proofreading polymerase and one non-proofreading polymerase. Specifically, *Wang* states:

> Currently, PCR amplification of long DNA fragments requires the use of an enzyme mixture containing both a non-proofreading polymerase (e.g. Taq or DyNAzyme II) and a small amount of proofreading polymerase *(e.g.* Pfu or Deep Vent). We have compared a single fusion enzyme, Pfu-Sso7d, to one of the high performance, long PCR enzymes DyNAzyme EXT (from Finnzymes) in long PCR, and demonstrated that Pfu-Sso7d outperforms DyNAzyme EXT, especially with limited extension time.

Wang, pages 39-40. Furthermore, *Wang* teaches that the polymerase fusion outperforms the non-fusion polymerase mixture.

Thus, as to claims 47 and 48, *Wang* teaches away from adding a second DNA polymerase to a polymerase fusion because the polymerase fusion of *Wang* outperforms the state of the art polymerase mixtures and was intended as a substitute for such mixtures. The Examiner asserts that "it is expected that a blend of fusion polymerase Pfu-Sso7d and Pfu polymerase would have functioned in the claimed method, as *Wang* teaches both polymerases function at high pH (See example 6-1)." Final Office Action at page 6. Appellant's response is that *Wang* does not teach that either the Pfu polymerase or the Pfu-Sso7d polymerase fusion functions at a pH above 9. Moreover, the evidence of record shows that Pfu loses its activity at a pH above 9 and that one of skill in the art, as of the filing date of Appellant's application, would have expected a Pfu-Sso7d polymerase fusion to similarly loses its activity at a pH above 9. *See* Declaration at ¶¶ 13-16.

Wang further teaches away from claim 48, which recites that the second DNA polymerase is Pfu, because *Wang* teaches that the mixture requires a proofreading polymerase and a non-proofreading polymerase. The blend in claim 48, on the other hand, comprises two proofreading polymerases, a Pfu-Sso7d polymerase fusion and Pfu. *Wang* does not teach or suggest using two proofreading polymerases in a mixture.

Accordingly, for these additional reasons, Appellant requests that the Board reverse the Examiner's rejection of claims 47 and 48 as unpatentable under 35 U.S.C. § 103 over *Wang*.

B. *Wang* in View of *Sanger* Does Not Render Obvious Claims 5 and 6

The Examiner rejects claims 5 and 6 under 35 U.S.C. § 103(a) as allegedly obvious over *Wang* in combination with *Sanger*. Final Office Action at page 5. Appellant respectfully requests reversal of this rejection.

To establish a *prima facie* case of obviousness, the prior art reference (or references when combined) must teach or suggest all the claim limitations. *See* M.P.E.P. § 2142. Appellant submits that the combined teachings of the cited references do not teach all of the elements of the rejected claims. For the reasons discussed above, *Wang* fails to teach or suggest a pH from 9.3 to 12, as recited in claims 5 and 6. *Sanger* also fails to teach or suggest this element of the claims and thus fails to remedy the deficiencies of *Wang*.

Accordingly, Appellant submits that the combined teachings of *Wang* and *Sanger* fail to teach or suggest all elements of claims 5 and 6 and, thus, do not render those claims obvious. For at least this reason, Appellant requests that the Board reverse the rejection of claims 5 and 6 as unpatentable under 35 U.S.C. § 103 over *Wang* in view of *Sanger*.

C. *Wang* in View of *Dietrich* Does Not Render Claims 49-52 Obvious

The Examiner rejects claims 49-52 under 35 U.S.C. § 103(a) as allegedly obvious over *Wang* in view of *Dietrich*. Final Office Action at page 7. Appellant requests reversal of this rejection.

The Examiner acknowledges that "Wang does not specifically teach using the Pfu-Sso7d fusion polymerase protein at the pH range of 9.5 to 12." *Id.* The Examiner, however, asserts that

> it would have been obvious to one of ordinary skill in the art at the time the claimed invention was made to modify the methods for a modified polymerase of Wang by using a higher pH as suggested by Dietrich et al. with a reasonable expectation of success. The motivation to do so is provided by Dietrich et al. who teach recombinant modified polymerases can have more activity at pH 9.5 and can retain more than 80% of optimal activity over a broad pH range of 7 to 10.

Id. at 8.

As discussed above, it is improper for the Examiner to extrapolate a general teaching about DNA polymerases from a specific reference about a *Pab* DNA polymerase, particularly here, where the evidence of record shows that *Pfu* DNA polymerase loses its activity above pH 9. Declaration, ¶ 13. *Dietrich* cannot provide one of skill in the art with a reasonable expectation of success when the evidence of record shows that Pfu loses its activity above pH 9 and that one of skill in the art would similarly expect a pH above 9 to significantly impair the conditions for amplification when using a fusion protein comprising Pfu. Declaration, ¶ 16. *Dietrich*, therefore, is far less relevant, if relevant at all, than the evidence of record regarding Pfu DNA polymerase and fusion proteins comprising the same. Accordingly, in view of the totality of the evidence, *Dietrich* does not provide the motivation for raising the pH of the buffer used with *Wang*'s Pfu-Sso7d fusion polymerase to 9.3 or 9.5.

The Examiner further asserts that

> it would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to use the claimed pH of 9.5 and above as used by the applicant or in the range of pH 9.0 as used by Wang since these differences in pH would not be expected to greatly alter the conditions for amplification. One of ordinary skill in the art would have not expected that the activity of a DNA polymerase fusion would be completely lost at pH 9.5 when it was functional at pH 9.0.

Id.

Appellant's response is that based on the evidence of record, one of skill in the art would have expected the differences in the pH of *Wang* and the claimed pH to significantly impair the conditions for amplification. As discussed above, the Borns Declaration establishes that one of skill in the art would have expected that increasing the pH of the reaction buffer in Example 6-1 of *Wang* from 8.8 to above 9 would significantly impair the conditions for amplification with *Wang*'s Pfu-Sso7d fusion polymerase, just as it did for the wild type Pfu polymerase. Declaration, ¶ 16. The Examiner attempts to rebut this evidence by citing *Dietrich*, but the statements in *Dietrich* about the Pab DNA polymerase maintaining significant activity at pH 9.5-10 does not refute the evidence of record showing that the Pfu DNA polymerase loses its activity above pH 9 and has little to no activity at pH 9.5. Furthermore, *Gueguen*, which discloses pH dependency studies for Pab DNA polymerase and is cited in *Dietrich*, actually shows that Pab DNA polymerase does not maintain significant activity at pH 9.5-10 but rather undergoes a drastic reduction in activity at pH greater than 9, essentially losing activity altogether at pH 9.5.

Finally, the Examiner asserts:

> Routine optimization is not considered inventive and no evidence has been presented that the selection of pH 9.5 was other than routine, that the products resulting from the optimization have any unexpected properties, or that the results should be considered unexpected in any way as compared to the closest prior art of pH 9.0. As noted, a skilled artisan would expect a pH of 9.0 to have nearly identical properties in the amplification of nucleic acids. Thus, an ordinary practitioner would have recognized that the results could be adjusted to maximize the desired results especially in view of the teachings of Dietrich et al. as given above.

Final Office Action at page 9.

As discussed above, selection of a pH of 9.3 to 12 or 9.5 to 12 was not routine optimization because one of skill in the art would have expected the differences between the conventional pH used for Pfu-based PCR in *Wang* (about 8.3 to 8.8) and the pH recited in the

claims (9.3 to 12 or 9.5 to 12) to significantly impair the efficiency of PCR performance. *See* Declaration at ¶ 16.

For at least this reason, Appellant requests that the Board reverse the rejection of claims 49-52 as unpatentable under 35 U.S.C. § 103 over *Wang* in combination with *Dietrich*.

CONCLUSION

For the reasons given above, pending claims 1-10, 13, 15, 25-29, and 40-52 are allowable and reversal of the Examiner's rejection is respectfully requested.

To the extent any extension of time under 37 C.F.R. § 1.136, not accounted for above, is required to obtain entry of this Appeal Brief, such extension is hereby respectfully requested. If there are any fees due that are not enclosed, including any fees required for an extension of time under 37 C.F.R. § 1.136, please charge such fees to Deposit Account No. 50-3740.

Respectfully submitted,
Michael BORNS

Date: 6 April 2009

By: / Timothy B. Donaldson/
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APPENDIX 1
CLAIMS ON APPEAL

1. (Rejected) A method for DNA synthesis at high pH, comprising: a) contacting a DNA polymerase fusion with a nucleic acid template under conditions of high pH, and b) effecting template dependent synthesis of DNA, wherein said high pH ranges from 9.3 to 12, and wherein said DNA polymerase fusion comprises wild type *Pyrococcus furiosus* polymerase I fused to *Sulfolobus solfataricus* SSso7d protein, wherein said DNA polymerase fusion functions as a DNA polymerase.

2. (Rejected) The method of claim 1, further comprising contacting a PCR enhancing factor and/or an additive with said DNA polymerase fusion and said nucleic acid template.

3. (Rejected) A method for the cloning of a DNA synthesis product, at high pH, wherein said high pH ranges from 9.3 to 12, comprising:

a) providing a DNA polymerase fusion comprising wild-type *Pyrococcus furiosus* polymerase I fused to *Sulfolobus solfataricus* SSso7d protein, wherein said DNA polymerase fusion functions as a DNA polymerase;

b) contacting said DNA polymerase fusion with a nucleic acid temp1ate under conditions of said high pH so as to effect the template dependent synthesis of a DNA synthesis product, and

c) inserting said synthesized DNA product into a cloning vector, thereby cloning said synthesized DNA product.

4. (Rejected) The method of claim 3, further comprising contacting a PCR enhancing factor and/or an additive with said DNA polymerase fusion and said nucleic acid template in step

(b).

5. (Rejected) A method for sequencing DNA at high pH, wherein said high pH ranges from 9.3 to 12, comprising the steps of:

(a) contacting a template DNA strand with a sequencing DNA primer;

(b) contacting said DNA of step (a) with a DNA polymerase fusion comprising wild-type *Pyrococcus furiosus* polymerase I fused to *Sulfolobus solfataricus* SSso7d protein, wherein said DNA polymerase fusion functions as a DNA polymerase, deoxyribonucleoside triphosphates, and a chain-terminating nucleotide analog;

(c) incubating the mixture of step (b) under conditions sufficient to synthesize at said high pH a random population of DNA molecules complementary to said DNA molecule, wherein said synthesized DNA molecules are shorter in length than said first DNA molecule and wherein said synthesized DNA molecules comprise a terminator nucleotide at their 5' termini; and

(d) separating said synthesized DNA molecules by size so that at least a part of the nucleotide sequence of said first DNA molecule can be determined.

6. (Rejected) The method of claim 5, further comprising a PCR enhancing factor and/or an additive.

7. (Rejected) A method of linear or exponential PCR amplification at high pH, wherein said high pH ranges from 9.3 to 12, for site-directed or random mutagenesis comprising the step of: incubating a reaction mixture comprising a nucleic acid template, at least one PCR primers, and a DNA polymerase fusion comprising wild-type *Pyrococcus furiosus* polymerase I fused to

Sulfolobus solfataricus SSso7d protein, wherein said DNA polymerase fusion functions as a DNA polymerase, under conditions which permit amplification of said nucleic acid template at said high pH by said fusion to produce a mutated amplified product.

8. (Rejected) The method of claim 7, further comprising a PCR enhancing factor and/or an additive.

9. (Rejected) A method of reverse transcriptase PCR at high pH, wherein said high pH ranges from 9.3 to 12, comprising the step of incubating a reaction mixture comprising a nucleic acid template, at least one PCR primer, and a DNA polymerase fusion comprising wild-type *Pyrococcus furiosus* polymerase I fused to *Sulfolobus solfataricus* SSso7d protein, wherein said DNA polymerase fusion functions as a DNA polymerase, under reaction conditions which permit amplification of said nucleic acid template at said high pH by said fusion to produce an amplified product.

10. (Rejected) The method of claim 9, further comprising a PCR enhancing factor and/or an additive.

11. (Cancelled)

12. (Withdrawn) The method of claim 11, wherein said DNA polymerase fusion comprises a Glycine to Proline substitution at amino acid position 387 (G387P) and has reduced DNA polymerization activity.

13. (Rejected) The method of claim 1, 3, 5, 7 or 9, wherein said DNA polymerase fusion comprises reduced base analog detection activity relative to wild-type *Pyrococcus furiosus* polymerase I under identical reaction conditions.

14. (Withdrawn) The method of claim 13, wherein said DNA polymerase fusion comprises reduced base analog detection activity and a mutation at position V93, wherein said mutation is a Valine to Arginine substitution, a Valine to Glutamic acid substitution, a Valine to Lysine substitution, a Valine to Aspartic acid substitution or a Valine to Asparagine substitution.

15. (Rejected) The method of claim 11 wherein said DNA polymerase fusion has reduced base analog detection activity relative to wild-type *Pyrococcus furiosus* polymerase I under identical reaction conditions.

16. (Withdrawn) The method of claim 12, wherein said DNA polymerase fusion comprises reduced base analog detection activity relative to wild-type *Pyrococcus furiosus* polymerase I under identical reaction conditions.

17. (Withdrawn) The method of claim 11, wherein said DNA polymerase fusion further comprises a mutation at position V93, wherein said mutation is a Valine to Arginine substitution, a Valine to Glutamic acid substitution, a Valine to Lysine substitution, a Valine to Aspartic acid substitution or a Valine to Asparagine substitution that confers a reduced base analog detection activity phenotype to said chimeric DNA polymerase.

18. (Withdrawn) The method of claim 12, wherein said DNA polymerase fusion further comprises a mutation at position V93, wherein said mutation is a Valine to Arginine substitution, a Valine to Glutamic acid substitution, a Valine to Lysine substitution, a Valine to Aspartic acid substitution or a Valine to Asparagine substitution that confers a reduced base analog detection activity phenotype to said chimeric DNA polymerase.

19. (Cancelled)

20. (Cancelled)

21. (Withdrawn) The method of claim 13, wherein said DNA polymerase fusion further comprises a Glycine to Proline substitution at amino acid position 387 (G387P) that confers a reduced DNA polymerization phenotype to said chimeric DNA polymerase.

22. (Withdrawn) The method of claim 14, wherein said DNA polymerase fusion further comprises a Glycine to Proline substitution at amino acid position 387 (G387P) that confers a reduced DNA polymerization phenotype to said chimeric DNA polymerase.

23. (Withdrawn) The method of claim 1, 3, 5, 7 or 9, wherein said DNA polymerase fusion further comprises an Aspartate to alanine substitution at amino acid 141 (Dl41A) and a Glutamic acid to Alanine substitution at amino acid position 143 (D141A/E143A) that renders said chimeric DNA polymerase 3'-5' exonuclease deficient.

24. (Withdrawn) The method of claim 13, wherein said DNA polymerase fusion with reduced base analog detection activity further comprises an Aspartate to alanine substitution at amino acid 141 (D141A) and a Glutamic acid to Alanine substitution at amino acid position 143 (D141A/E143A) that renders said chimeric DNA polymerase 3'-5' exonuclease deficient.

25. (Rejected) The method of claim 1, 3, 5, 7 or 9, wherein said DNA polymerase fusion comprises a wild type, mutant or chemically modified DNA polymerase.

26. (Rejected) The method of claim 1, 3, 5, 7, or 9, wherein said DNA polymerase fusion is a proofreading polymerase.

27. (Rejected) The method of claim 26, wherein said proofreading polymerase comprises wild-type *Pyrococcus furiosus* polymerase I.

28. (Rejected) The method of claim 1, 3, 5, 7, or 9, wherein said DNA polymerase fusion comprises an increase, as compared to said wild type *Pyrococcus furiosus* polymerase I, in at least one activity selected from the group consisting of: processivity, proofreading, fidelity, DNA binding activity, strand displacement activity, polymerase activity, nucleotide binding and recognition, efficiency, template length amplification capability, GC-rich target amplification efficiency, specificity, thermostability, intrinsic hot start capability, or salt resistance.

29. (Rejected) The method of claim 1, 3, 5, 7 or 9, wherein said DNA polymerase fusion

comprises a reduction, as compared to said wild type *Pyrococcus furiosus* polymerase I, in at least one activity selected from the group consisting of: amplification slippage on templates with tri-nucleotide repeat stretches, extension time in a PCR reaction or amplification cycles in a PCR reaction.

Claims 30-39. (Cancelled)

40. (Rejected) The method of any one of claims 1, 3, 5, 7, and 9, wherein said DNA polymerase fusion is encoded by SEQ ID NO: 126 and has an amino acid sequence of SEQ ID NO:127.

41. (Rejected) The method of claim 29, wherein the activity is extension time in a PCR reaction.

42. (Rejected) The method of claim 1, wherein said high pH ranges from 9.5 to 12.

43. (Rejected) The method of claim 3, wherein said high pH ranges from 9.5 to 12.

44. (Rejected) The method of claim 5, wherein said high pH ranges from 9.5 to 12.

45. (Rejected) The method of claim 7, wherein said high pH ranges from 9.5 to 12.

46. (Rejected) The method of claim 9, wherein said high pH ranges from 9.5 to 12.

47. (Rejected) The method of claim 42, wherein the DNA polymerase fusion is part of a blend comprising a second DNA polymerase.

48. (Rejected) The method of claim 47, wherein the second DNA polymerase is *Pfu*.

49. (Rejected) The method of claim 41, wherein the extension time is decreased by at least 15 seconds as compared to the extension time observed under the same conditions with the wild type Pfu polymerase.

50. (Rejected) The method of claim 49, wherein the extension time is decreased by at least 45 seconds as compared to the extension time observed under the same conditions with the wild type Pfu polymerase.

51. (Rejected) The method of claim 49, wherein said high pH ranges from 9.5 to 12.

52. (Rejected) The method of claim 50, wherein said high pH ranges from 9.5 to 12.

APPENDIX 2

EVIDENCE SUPPORTING APPEAL BRIEF

In addition to the specification, Appellant relies on the following evidence in support of this appeal brief:

- Declaration Under 37 C.F.R. §1.132 of Michael Borns submitted into evidence as Attachment 1 to Amendment and Request for Reconsideration filed 17 December 2007; and
- Gueguen *et al.*, Eur. J. Biochem. 268, 5961-69 (2001) submitted into evidence as Attachment A to Amendment After Final Rejection Under 37 C.F.R. § 1.116 filed 8 December 2008.

Copies of the Declaration and Gueguen *et al.* are being submitted with the Appeal Brief under a separate transmittal letter.

APPENDIX 3
DECISIONS IN PROCEEDINGS RELATED TO APPEAL

There are no decisions in proceedings related to this appeal.

PATENT
Attorney Docket No. 10070485-02-US
Customer No. 27,495

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent Application of

Applicant: Michael BORNS

Application No.: 10/805,650

Filing Date: 19 March 2004

Group Art Unit: 1637

Confirmation No.: 9645

Examiner: M. STAPLES

Title: DNA POLYMERASE FUSIONS AND USES THEREOF

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

SUBMISSION OF EVIDENCE SUPPORTING APPEAL BRIEF

As indicated in Appendix 2 of the Appeal Brief filed concurrently herewith, Applicant submits copies of the following evidence in support of this appeal brief:

- Declaration Under 37 C.F.R. §1.132 of Michael Borns submitted into evidence as Attachment 1 to Amendment and Request for Reconsideration filed 17 December 2007 (EXHIBIT 1); and
- Gueguen *et al.*, Eur. J. Biochem. 268, 5961-69 (2001) submitted into evidence as Attachment A to Amendment After Final Rejection Under 37 C.F.R. § 1.116 filed 8 December 2008 (EXHIBIT 2).

Respectfully submitted,
Michael BORNS

Date: 6 April 2009

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EXHIBIT 1

PATENT
Attorney Docket No. STG-167
Customer No. 27,495

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent Application of

Applicant: Michael BORNS

Application No.: 10/805,650

Filing Date: 19 March 2004

Group Art Unit: 1637

Confirmation No.: 9645

Examiner: M. STAPLES

Title: DNA POLYMERASE FUSIONS AND USES THEREOF

Mail Stop: RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. §1.132

I, Michael Borns declare that:

1. I have read U.S. Application No. 10/805,650, filed 19 March 2004, as well as the pending claims (Attachment A).

2. I am the inventor of the subject matter claimed in U.S. Application No. 10/805,650.

3. I am informed and believe that U.S. Application No. 10/805,650 claims the benefit of U.S. Provisional Application No. 60/457,426, filed 25 March 2003.

4. I graduated from the University of Oregon in 1988 with a B.S. in molecular biology. I worked for a year in a Drosophila lab studying the genes and proteins involved in retinal degeneration in the larval development of the fruit fly. From 1989-1990 I worked at Children's

Hospital doing cancer research. In 1990, I started working for a company called Xoma in Santa Monica, California as a research associate. At Xoma my research focused on cloning genes, and I performed standard molecular biology experiments, such as PCR, Southern blotting, and Northern blotting. I left Xoma around 1992 and started working for One Lamda in Canoga Park, California as a senior research associate. I worked at One Lambda for five years studying DNA polymerases, such as Taq, and developing PCR-based tissue typing kits. In 1997, I changed jobs and started working for Stratagene in San Diego, California as a senior research associate. I am currently employed by Stratagene, an Agilent Technologies Company, and hold the title of research scientist expert. During my ten years at Stratagene, I have worked in the enzyme group, studying DNA polymerases, including Pfu polymerase, and developing PCR kits.

5. The statements made in this declaration are based on my 15 years experience working with DNA polymerases, including the last 10 years working with the wild type Pfu DNA polymerase.

6. I have read Item 12 (pages 6-11) of the Office Action mailed 17 August 2007 and understand that the Examiner has rejected certain claims as being unpatentable over "Wang" (WO 01/082501).

7. As of at least 25 March 2003, the pH of the buffering component in the standard PCR reaction buffers for wild type Pfu DNA polymerase was 8.3 to 8.8.

8. In general, when the buffering component is added to the PCR reaction buffer, the final pH of the PCR reaction buffer will be slightly lower than the pH of the buffering component.

9. With standard wild type DNA polymerases, such as wild type Pfu DNA polymerase, as pH increases above 9, there is an inverse relationship with PCR performance. In other words, as the pH increases above 9, PCR performance decreases.

10. Using a wild type Pfu DNA polymerase in a reaction buffer having a pH above 9 impairs the efficiency of PCR performance.

11. Attachment B shows the results of an experiment I performed in October 2007 where I examined the effects of increasing pH on wild type Pfu DNA polymerase (Pfu Turbo) for the amplification of a 6 kb human beta globin DNA target. Lane 1 is a 1 kb DNA marker. Lanes 2, 3, and 4 are with pH 8.3, 8.5, and 8.8, respectively. Lanes 5 and 6 are with pH 9.5 and 10, respectively.

12. In this experiment, a 6 kb human globin genomic DNA target sequence was amplified with 2.5 units of wild type Pfu DNA polymerase and 100 ng human genomic DNA per 50 μl reaction in each lane. A 1 minute per kb extension time was used for a total extension time of 6 minutes.

13. As shown in Attachment B, wild type Pfu DNA polymerase efficiently amplifies target DNA in a reaction buffer ranging from pH 8.3 to 8.8. However, when the pH of the reaction buffer was increased above 9, the efficiency of the Pfu amplification activity dropped

dramatically. The results in Attachment B are consistent with my experience working with wild type Pfu DNA polymerase as of 25 March 2003.

14. In contrast, with the DNA polymerase fusions of this invention, increasing the pH of the reaction buffer above 9 actually enhances—not reduces—the efficiency of PCR performance.

15. I have read the Wang reference cited in the 17 August 2007 Office Action. Example 6.1 of Wang discloses using wild type Pfu polymerase and a Pfu-Sso7d fusion polymerase with a reaction buffer containing a 20 mM Tris-HCl buffering component having a pH of 8.8. It appears that the reaction buffer used by Wang was the commercially available Pfu reaction buffer from Stratagene.

16. Based on my experience working with wild type Pfu DNA polymerase, I would have expected as of at least 25 March 2003 that increasing the pH of the Pfu reaction buffer disclosed in Example 6.1 of Wang above a pH of 9 would significantly impair the conditions for amplification with the wild type Pfu DNA polymerase. Without considering any of the work disclosed in U.S. Application No. 10/805,650, and based solely on my experience working with wild type Pfu DNA polymerase, I would have similarly expected as of at least 25 March 2003 that increasing the pH of the Pfu reaction buffer disclosed in Example 6.1 of Wang above a pH of 9 would significantly impair the conditions for amplification with Wang's Pfu-Sso7d fusion polymerase. Therefore, as of at least 25 March 2003, I would not have been motivated to increase the pH of the reaction buffer disclosed in Example 6.1 of Wang as a matter of routine optimization for either the wild type Pfu DNA polymerase or the Pfu-Sso7d fusion polymerase,

because I would have expected that doing so would significantly impair the efficiency of their PCR performance. In addition, as of 25 March 2003, I would not have been motivated to increase the pH of the reaction buffer disclosed in Example 6.1 of Wang as a matter of routine optimization because Wang appears to have been using the commercially available Pfu reaction buffer from Stratagene, which as a commercial product, would have already been optimized.

17. The expectations referred to in the preceding paragraph of my declaration are based solely on my experience working with wild type Pfu DNA polymerase and other wild type DNA polymerases and do not take into account any of the work disclosed in the instant application, which was not publicly available as of the 25 March 2003 filing date of U.S. Provisional Application No. 60/457,426.

18. Thus, it was surprising when I discovered that increasing the pH of the reaction buffer above 9 enhances, rather than impairs, the PCR performance efficiency of the DNA polymerase fusions in U.S. Application No. 10/805,650, and blends comprising the same. *See* Figures 1-9 of the '650 application. This unexpected result was duly noted in the application. For example, the application notes that "PCR reactions using the high pH 10.0 and 11.8 reaction buffers were dramatically superior to the 1.5X cloned *Pfu* buffer, further demonstrating the enhancing effects of high pH for PCR amplification with *Pfu*-Sso7d (figure 3)." U.S. Application No. 10/805,650 at pages 80-81.

Attorney Docket No.: STG-167
U.S. Application No. 10/805,650
Customer No.: 27,495

19. The undersigned further declares that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing therefrom.

By: ____________________

Michael Borns

Dated: 12/17/07

ATTACHMENT A

PENDING CLAIMS

1. (Previously presented) A method for DNA synthesis at high pH, comprising: a) contacting a DNA polymerase fusion with a nucleic acid template under conditions of high pH, and b) effecting template dependent synthesis of DNA, wherein said high pH ranges from 9.3 to 14, and wherein said DNA polymerase fusion comprises wild type *Pyrococcus furiosus* polymerase I fused to *Sulfolobus solfataricus* SSso7d protein, wherein said DNA polymerase fusion functions as a DNA polymerase.

2. (Previously presented) The method of claim 1, further comprising contacting a PCR enhancing factor and/or an additive with said DNA polymerase fusion and said nucleic acid template.

3. (Previously presented) A method for the cloning of a DNA synthesis product, at high pH, wherein said high pH ranges from 9.3 to 14, comprising:

a) providing a DNA polymerase fusion comprising wild-type *Pyrococcus furiosus* polymerase I fused to *Sulfolobus solfataricus* SSso7d protein, wherein said DNA polymerase fusion functions as a DNA polymerase;

b) contacting said DNA polymerase fusion with a nucleic acid temp1ate under conditions of said high pH so as to effect the template dependent synthesis of a DNA synthesis product, and

c) inserting said synthesized DNA product into a cloning vector, thereby cloning said synthesized DNA product.

4. (Previously presented) The method of claim 3, further comprising contacting a PCR

enhancing factor and/or an additive with said DNA polymerase fusion and said nucleic acid template in step (b).

5. (Previously presented) A method for sequencing DNA at high pH, wherein said high pH ranges from 9.3 to 14, comprising the steps of:

(a) contacting a template DNA strand with a sequencing DNA primer;

(b) contacting said DNA of step (a) with a DNA polymerase fusion comprising wild-type *Pyrococcus furiosus* polymerase I fused to *Sulfolobus solfataricus* SSso7d protein, wherein said DNA polymerase fusion functions as a DNA polymerase, deoxyribonucleoside triphosphates, and a chain-terminating nucleotide analog;

(c) incubating the mixture of step (b) under conditions sufficient to synthesize at said high pH a random population of DNA molecules complementary to said DNA molecule, wherein said synthesized DNA molecules are shorter in length than said first DNA molecule and wherein said synthesized DNA molecules comprise a terminator nucleotide at their 5' termini; and

(d) separating said synthesized DNA molecules by size so that at least a part of the nucleotide sequence of said first DNA molecule can be determined.

6. (Original) The method of claim 5, further comprising a PCR enhancing factor and/or an additive.

7. (Previously presented) A method of linear or exponential PCR amplification at high pH, wherein said high pH ranges from 9.3 to 14, for site-directed or random mutagenesis comprising the step of: incubating a reaction mixture comprising a nucleic acid template, at least

one PCR primers, and a DNA polymerase fusion comprising wild-type *Pyrococcus furiosus* polymerase I fused to *Sulfolobus solfataricus* SSso7d protein, wherein said DNA polymerase fusion functions as a DNA polymerase, under conditions which permit amplification of said nucleic acid template at said high pH by said fusion to produce a mutated amplified product.

8. (Original) The method of claim 7, further comprising a PCR enhancing factor and/or an additive.

9. (Previously presented) A method of reverse transcriptase PCR at high pH, wherein said high pH ranges from 9.3 to 14, comprising the step of incubating a reaction mixture comprising a nucleic acid template, at least one PCR primer, and a DNA polymerase fusion comprising wild-type *Pyrococcus furiosus* polymerase I fused to *Sulfolobus solfataricus* SSso7d protein, wherein said DNA polymerase fusion functions as a DNA polymerase, under reaction conditions which permit amplification of said nucleic acid template at said high pH by said fusion to produce an amplified product.

10. (Original) The method of claim 9, further comprising a PCR enhancing factor and/or an additive.

11. (Cancelled)

12. (Withdrawn) The method of claim 11, wherein said DNA polymerase fusion comprises a Glycine to Proline substitution at amino acid position 387 (G387P) and has reduced DNA polymerization activity.

13. (Previously presented) The method of claim 1, 3, 5, 7 or 9, wherein said DNA

polymerase fusion comprises reduced base analog detection activity relative to wild-type *Pyrococcus furiosus* polymerase I under identical reaction conditions.

14. (Withdrawn) The method of claim 13, wherein said DNA polymerase fusion comprises reduced base analog detection activity and a mutation at position V93, wherein said mutation is a Valine to Arginine substitution, a Valine to Glutamic acid substitution, a Valine to Lysine substitution, a Valine to Aspartic acid substitution or a Valine to Asparagine substitution.

15. (Previously presented) The method of claim 11 wherein said DNA polymerase fusion has reduced base analog detection activity relative to wild-type *Pyrococcus furiosus* polymerase I under identical reaction conditions.

16. (Withdrawn – previously presented) The method of claim 12, wherein said DNA polymerase fusion comprises reduced base analog detection activity relative to wild-type *Pyrococcus furiosus* polymerase I under identical reaction conditions.

17. (Withdrawn) The method of claim 11, wherein said DNA polymerase fusion further comprises a mutation at position V93, wherein said mutation is a Valine to Arginine substitution, a Valine to Glutamic acid substitution, a Valine to Lysine substitution, a Valine to Aspartic acid substitution or a Valine to Asparagine substitution that confers a reduced base analog detection activity phenotype to said chimeric DNA polymerase.

18. (Withdrawn) The method of claim 12, wherein said DNA polymerase fusion further comprises a mutation at position V93, wherein said mutation is a Valine to Arginine substitution, a Valine to Glutamic acid substitution, a Valine to Lysine substitution, a Valine to Aspartic acid

substitution or a Valine to Asparagine substitution that confers a reduced base analog detection activity phenotype to said chimeric DNA polymerase.

19. (Cancelled)

20. (Cancelled)

21. (Withdrawn) The method of claim 13, wherein said DNA polymerase fusion further comprises a Glycine to Proline substitution at amino acid position 387 (G387P) that confers a reduced DNA polymerization phenotype to said chimeric DNA polymerase.

22. (Withdrawn) The method of claim 14, wherein said DNA polymerase fusion further comprises a Glycine to Proline substitution at amino acid position 387 (G387P) that confers a reduced DNA polymerization phenotype to said chimeric DNA polymerase.

23. (Withdrawn) The method of claim 1, 3, 5, 7 or 9, wherein said DNA polymerase fusion further comprises an Aspartate to alanine substitution at amino acid 141 (Dl41A) and a Glutamic acid to Alanine substitution at amino acid position 143 (D141A/E143A) that renders said chimeric DNA polymerase 3'-5' exonuclease deficient.

24. (Withdrawn) The method of claim 13, wherein said DNA polymerase fusion with reduced base analog detection activity further comprises an Aspartate to alanine substitution at amino acid 141 (D141A) and a Glutamic acid to Alanine substitution at amino acid position 143 (D141A/E143A) that renders said chimeric DNA polymerase 3'-5' exonuclease deficient.

25. (Original) The method of claim 1, 3, 5, 7 or 9, wherein said DNA polymerase fusion comprises a wild type, mutant or chemically modified DNA polymerase.

26. (Previously presented) The method of claim 1, 3, 5, 7, or 9, wherein said DNA polymerase fusion is a proofreading polymerase.

27. (Previously presented) The method of claim 26, wherein said proofreading polymerase comprises wild-type *Pyrococcus furiosus* polymerase I.

28. (Previously presented) The method of claim 1, 3, 5, 7, or 9, wherein said DNA polymerase fusion comprises an increase, as compared to said wild type *Pyrococcus furiosus* polymerase I, in at least one activity selected from the group consisting of: processivity, proofreading, fidelity, DNA binding activity, strand displacement activity, polymerase activity, nucleotide binding and recognition, efficiency, template length amplification capability, GC-rich target amplification efficiency, specificity, thermostability, intrinsic hot start capability, or salt resistance.

29. (Previously presented) The method of claim 1, 3, 5, 7 or 9, wherein said DNA polymerase fusion comprises a reduction, as compared to said wild type *Pyrococcus furiosus* polymerase I, in at least one activity selected from the group consisting of: amplification slippage on templates with tri-nucleotide repeat stretches, extension time in a PCR reaction or amplification cycles in a PCR reaction.

30. (Cancelled)

31. (Withdrawn – previously presented) A kit for performing at high pH, wherein said high pH ranges from 9.3 to 14, a method selected from the group consisting of: DNA synthesis; cloning of a DNA synthesis product; sequencing DNA; RT PCR; and linear or exponential PCR

amplification comprising a DNA polymerase fusion and packaging materials therefore.

32. (Withdrawn – previously presented) The kit of claim 31, further comprising a high pH buffer, wherein said high pH buffer has a pH which ranges from 9.3 to 14.

33. (Withdrawn) The kit of claim 31, further comprising a PCR enhancing factor and/or an additive.

34. (Withdrawn – previously presented) A composition for any one of DNA synthesis, cloning of a DNA synthesis product at high pH, sequencing DNA, linear or exponential PCR amplification for site directed or random mutagenesis, RT-PCR comprising a DNA polymerase fusion and a high pH buffer, wherein said high pH buffer has a pH which ranges from 9.3 to 14.

35. (Withdrawn – previously presented) A composition for DNA synthesis, comprising a DNA polymerase fusion and a high pH DNA synthesis buffer, wherein said high pH DNA synthesis buffer has a pH which ranges from 9.3 to 14.

36. (Withdrawn – previously presented) A composition for cloning of a DNA synthesis product, comprising a DNA polymerase fusion and a high pH DNA cloning buffer, wherein said high pH cloning buffer has a pH which ranges from 9.3 to 14.

37. (Withdrawn – previously presented) A composition for sequencing DNA, comprising a DNA polymerase fusion and a high pH DNA sequencing buffer, wherein said high pH sequencing buffer has a pH which ranges from 9.3 to 14.

38. (Withdrawn – previously presented) A composition for linear or exponential PCR amplification for site directed or random mutagenesis, or for RT-PCR comprising a DNA

polymerase fusion and a high pH PCR reaction buffer, wherein said high pH PCR reaction buffer has a pH which ranges from 9.3 to 14.

39. (Withdrawn) The composition of claims 34, 35, 36, 37 or 38, further comprising a PCR enhancing factor and/or an additive.

40. (Previously presented) The method of any one of claims 1, 3, 5, 7, and 9, wherein said DNA polymerase fusion is encoded by SEQ ID NO: 126 and has an amino acid sequence of SEQ ID NO:127.

41. (Previously presented) The method of claim 29, wherein the activity is extension time in a PCR reaction.

42. (New) The method of claim 1, wherein said high pH ranges from 9.5 to 12.

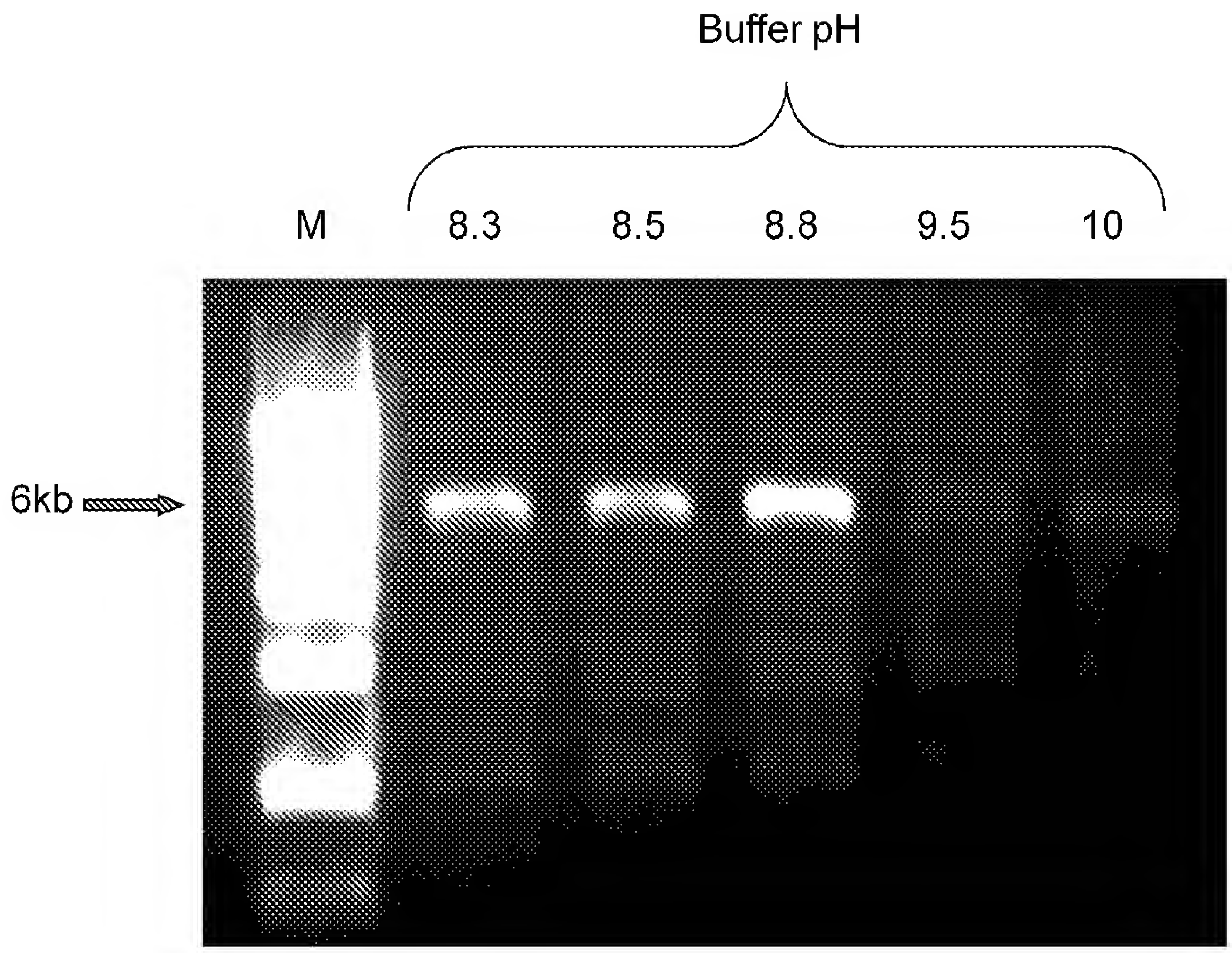
43. (New) The method of claim 3, wherein said high pH ranges from 9.5 to 12.

44. (New) The method of claim 5, wherein said high pH ranges from 9.5 to 12.

45. (New) The method of claim 7, wherein said high pH ranges from 9.5 to 12.

46. (New) The method of claim 9, wherein said high pH ranges from 9.5 to 12.

ATTACHMENT B



- 6kb human beta globin genomic DNA target (100ng human genomic DNA per 50µl reaction)
- 2.5 units of *PfuTurbo* per 50µl reaction
- 1 minute per kb extension time (6 minute total extension time)
- M (1kb ladder DNA marker)

EXHIBIT 2

Eur. J. Biochem. **268**, 5961–5969 (2001) © FEBS 2001

Characterization of two DNA polymerases from the hyperthermophilic euryarchaeon *Pyrococcus abyssi*

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The complete genome sequence of the hyperthermophilic archaeon *Pyrococcus abyssi* revealed the presence of a family B DNA polymerase (Pol I) and a family D DNA polymerase (Pol II). To extend our knowledge about euryarchaeal DNA polymerases, we cloned the genes encoding these two enzymes and expressed them in *Escherichia coli*. The DNA polymerases (Pol I and Pol II) were purified to homogeneity and characterized. Pol I had a molecular mass of ≈ 90 kDa, as estimated by SDS/PAGE. The optimum pH and Mg^{2+} concentration of Pol I were 8.5–9.0 and 3 mM, respectively. Pol II is composed of two subunits that are encoded by two genes arranged in tandem on the *P. abyssi* genome. We cloned these genes and purified the Pol II DNA polymerase from an *E. coli* strain coexpressing the cloned genes. The optimum pH and Mg^{2+} concentration of Pol II were 6.5 and 15–20 mM, respectively. Both *P. abyssi* Pol I and Pol II have associated 3′→5′ exonuclease activity although the exonuclease motifs usually found in DNA polymerases are absent in the archaeal family D DNA polymerase sequences. Sequence analysis has revealed that the small subunit of family D DNA polymerase and the Mre11 nucleases belong to the calcineurin-like phosphoesterase superfamily and that residues involved in catalysis and metal coordination in the Mre11 nuclease three-dimensional structure are strictly conserved in both families. One hypothesis is that the phosphoesterase domain of the small subunit is responsible for the 3′→5′ exonuclease activity of family D DNA polymerase. These results increase our understanding of euryarchaeal DNA polymerases and are of importance to push forward the complete understanding of the DNA replication in *P. abyssi*.

Keywords: *Pyrococcus abyssi*; archaea; DNA polymerase; DNA replication; exonuclease activity.

DNA polymerases play a leading role in the replication and maintenance of the genome and are central to the accurate transmission of genetic information from generation to generation. While our knowledge about DNA replication in eukarya and bacteria is quite advanced [1], limited information is available on the replication mechanism in archaea, the third major domain of life [2]. Recently, comparative genomics revealed that most archaeal proteins involved in DNA replication, transcription and translation are similar to those in eukarya, although the cellular appearance and organization of archaea are more similar to bacteria. Recent investigations [3,4] revealed that within the archaeota, euryarchaeota and crenarchaeota, the two major subdomains differ in their DNA replication mechanisms. The analysis of genome sequences indicated that many euryarchaea [5–7] possess a single family B (α-like) DNA-polymerase. In addition, a new heterodimeric DNA-polymerase (family D DNA polymerase) [8], with no significant homology to eukaryal or bacterial DNA polymerases, has been detected in the corresponding euryarchaeal genomes. No homologues of this heterodimeric DNA polymerase has so far been detected in the crenarchaeota kingdom and the genome sequencing of *Aeropyrum pernix* [9] and *Sulfolobus solfataricus* [10] suggests the absence of family D DNA polymerase homologues in crenarchaeotic cells. In contrast, the existence of two family B DNA polymerases in *A. pernix* [11] and *Pyrodictium occultum* [12] and of three family B DNA polymerases in *S. solfataricus* [13] indicates that several B-type DNA polymerases exist in the crenarchaeotal genomes. These findings confirm that the DNA replication mechanism of the euryarchaeal and crenarchaeal subdomains of archaea differs, and therefore opens the discussion of the evolution of DNA polymerases, a group of indispensable proteins that are central to the replication process. However, for a more precise interpretation of the evolutionary relationship between archaea and eukarya, additional genome sequences from crenarchaea require investigation.

P. abyssi is an anaerobic hyperthermophilic archaeon that belongs to the Thermococcales order within the euryarchaea subdomain. It was isolated from hydrothermal vents at a depth of 2000 meters in the south-west Pacific Ocean and grows optimally around 100 °C [14]. The complete genome sequence of *P. abyssi* revealed the presence of two DNA polymerases: a family B DNA polymerase (Pol I) and a family D DNA polymerase (Pol II). To increase our knowledge about euryarchaeal DNA polymerases, we cloned the genes for these two DNA polymerases, expressed them in

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Abbreviations: Pol I, DNA polymerase I; Pol II, DNA polymerase II; PCNA, proliferating cell nuclear antigen; RPA, replication protein A.

(Received 11 July 2001, revised 20 September 2001, accepted 26 September 2001)

Escherichia coli and characterized the two recombinant DNA polymerases.

MATERIALS AND METHODS

Organisms and growth conditions

P. abyssi (strain Orsay) was used in this study. The complete genome sequence of *P. abyssi* was determined at Genoscope (Evry, France). Sequences and annotations are available at http://www.genoscope.cns.fr/Pab/. The entire nucleotide sequence of *P. abyssi* was submitted to EMBL database under accession numbers CNSPAX01 to CNSPAX06. *E. coli* HMS174 (DE3), which harbors pLysS, was used as a host strain for overexpressing the cloned genes in the recombinant plasmids pPOLI, pPOLB and pPOLC. *E. coli* DH5α was also used as a host strain for the subcloning step. *E. coli* was grown in Luria–Bertani medium in a rotary shaker at 37 °C with ampicillin and/or kanamycin (final concentration of 100 μg·mL^{-1} or 30 μg·mL^{-1}, respectively).

Cloning of *P. abyssi* Pol I (*polI*) and Pol II genes (*polB, polC*)

Based on the sequence of *polI* (accession number P77916), primers were designed to amplify the DNA polymerase I gene (*polI*) from *P. abyssi* by the PCR on a DNA Thermal Cycler (Stratagene). The two primers (with *Nde*I and *Bam*HI restriction sites in bold) were as follows: POLI-1 (sense), 5′-CAGATTGGGTGGGG**CATATG**ATAATCGATGC-3′; and POLI-2 (antisense), 5′-CCCGA**GGATCC**TAGAACTTAAGCCATGCTCC-3′. The heterodimeric DNA Pol II from *P. abyssi* is composed of two subunits (PolB and PolC) whose genes (*polB* and *polC*) are arranged in tandem on the *P. abyssi* genome (Fig. 1). Based on the sequences of *polB* and *polC* (accession numbers F75199 and E75199, respectively), primers were designed to amplify separately the two DNA polymerase II genes. The two sets

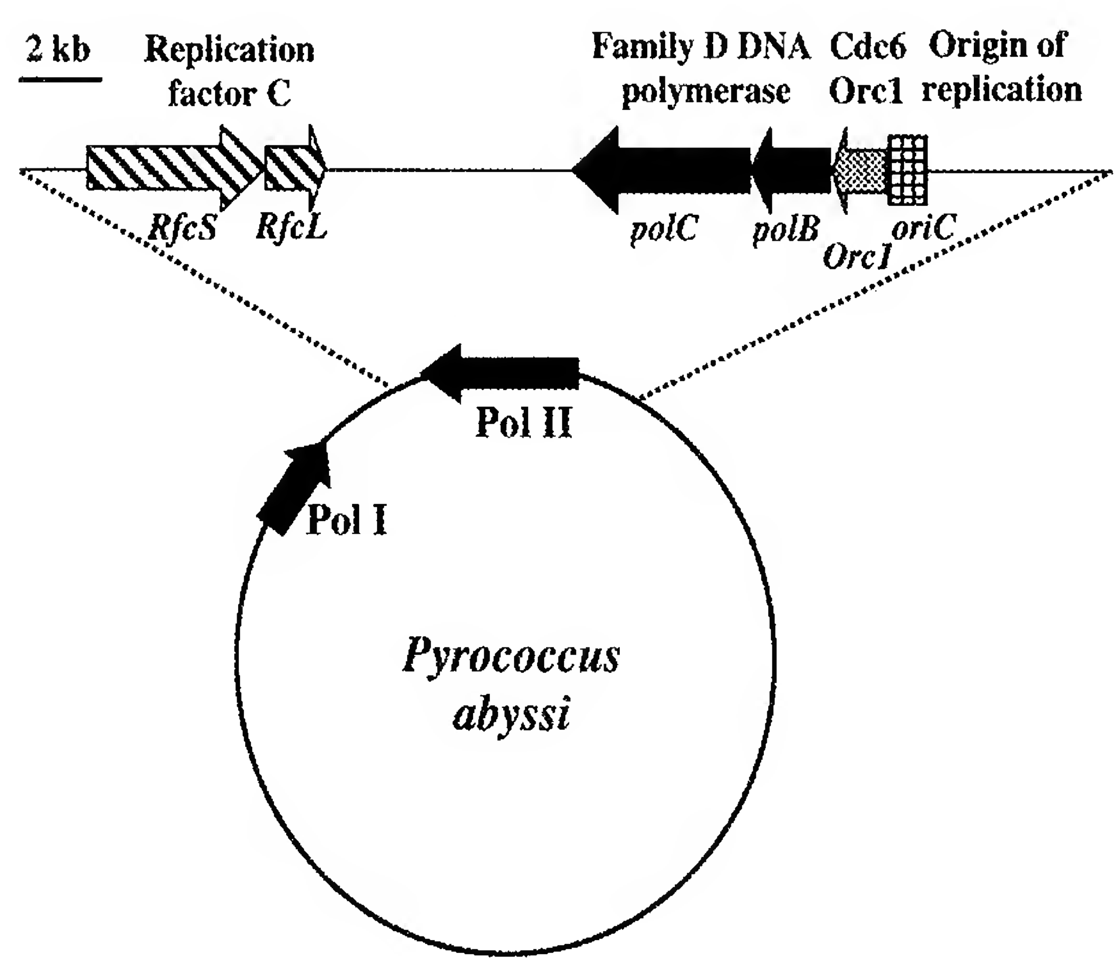

Fig. 1. Localization of the genes corresponding to Pol I and Pol II in the genome of *P. abyssi*. Genes located in the vicinity of Pol II that encode proteins that participate in DNA replication are shown. These include the origin of replication, the two subunits of the replication factor C and the Cdc6/Orc1 protein that is needed for the initiation of DNA replication.

of primers (with *Nde*I and *Bam*HI or *Nde*I and *Sal*I restriction sites in bold) were as follows: POLB1 (sense), 5′-CAAAGGAGGTTGCT**CATATG**GATGAATTGGTTAAGG-3′; POLB2 (antisense), 5′-TTCCTTTGGA**GGATCC**ATCAACACCACCCGCTG-3′; POLC1 (sense), 5′-AGCGGGTGGTG**CATATG**GAGCTTCCAAAGG-3′; POLC2 (antisense), 5′-TCGATGAGTACTAAG**GTCGAC**TTAGTAGATTTCACG-3′. In addition to the DNA template from *P. abyssi* and the primers, the 50-μL reaction mixture contained 10 nmol dNTPs, *Pfu* DNA polymerase buffer and 2 U *Pfu* DNA polymerase (Promega) and was subjected to 20 cycles of amplification (30 s at 94 °C, 30 s at 50 °C and 3 min at 72 °C). A PCR product of the expected size for *polI* was digested with *Nde*I and *Bam*HI and cloned into pET-26b + (Novagen, Inc.), resulting in pPOLI, and transformed into *E. coli* DH5α according to standard procedures [15]. The *polC* gene, encoding the large subunit of the Pol II, was shown to contain an intein coding region, which is an intervening sequence spliced out as a protein and not as a mRNA [22]. To prevent a possible toxic effect of the intein on *E. coli*, the gene encoding intein was deleted by splicing by overlap extension PCR [16] with the following primers: POLC3: 5′-GAGGAGAAACTGTGATGGAGATGAAGACGCTG-3′ and POLC4: 5′-CTCCATCACAGTTTCTCCTCTTCGCAGCGTGG-3′, resulting in the *polC2* gene. The two fragments containing *polB and polC2* were digested with *Nde*I and *Bam*HI or *Nde*I and *Sal*I, respectively, and cloned into pARHS [17] or pET-26b+ (Novagen), respectively, resulting in pPOLB and pPOLC, which were transformed into *E. coli* DH5α according to standard procedures [15].

Expression and purification of *P. abyssi* Pol I and Pol II

The recombinant plasmid pPOL1 containing *polI* gene was used for transformation of *E. coli* HMS174 (DE3)pLysS. In the case of Pol II, the two recombinant plasmids, pPOLB and pPOLC, were used to cotransform *E. coli* HMS174 (DE3)pLysS. Overnight cultures of *E. coli* HMS174 (DE3)pLysS harboring pPOL1 and *E. coli* HMS174 (DE3)pLysS harboring pPOLB and pPOLC were diluted 1 : 20 and grown until D_{600} reached 0.6. The two cultures were induced with 1 mM of isopropyl thio-β-D-galactoside (IPTG) for 16 h. Cells were harvested by centrifugation. The Pol I culture was resuspended in 50 mM phosphate buffer (pH 7.5 containing 1 mM EDTA, 10 mM 2-mercaptoethanol and 200 μg·mL^{-1} lysozyme) and the Pol II culture was resuspended in 50 mM Tris/HCl buffer (pH 8 containing 10% glycerol and 10 mM 2-mercaptoethanol). The cells were disrupted by sonication using a Vibracell sonifier (375 W, 40% amplitude). Cell debris were removed by centrifugation (10 000 ***g*** for 10 min). The resulting supernatants were heated for 30 min at 70 °C for Pol I and 30 min at 80 °C for Pol II and the precipitated proteins were removed by further centrifugation. The supernatants were subsequently dialyzed against buffer A (50 mM Tris/HCl, pH 8) for Pol I and buffer B (50 mM potassium phosphate, pH 6.5, 2 mM 2-mercaptoethanol, 10% glycerol) for Pol II. The dialysates were applied to a Resource-Q column (Pharmacia) that had been pre-equilibrated with the corresponding buffer (A for Pol I, B for Pol II) by use of an FPLC system (Pharmacia). Bound proteins were eluted by a linear gradient of NaCl (0–0.5 M

in buffer A or B). The active fractions were dialyzed against buffer A for Pol I and buffer B for Pol II and applied to a 5-mL heparin–Sepharose column (Hitrap Heparin, Pharmacia). The columns were developed with a linear gradient of 150 mM to 1 M NaCl in buffer C (50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 0.05% Triton X-100) for Pol I and a linear gradient of 150 mM to 650 mM in buffer B for Pol II.

Protein samples were analyzed by SDS/PAGE using the method of Laemmli [18]. Protein concentrations were determined by the method of Bradford, with BSA as the standard [19]. Protein bands were visualized by staining with Coomassie Brillant Blue R-250.

DNA polymerase assay

DNA polymerizing activity was assayed by measuring the incorporation of [*methyl*-^{3}H]dTMP into trichloroacetic acid insoluble material. The principle of the assay has been described previously [12,20]. In brief, the 20-µL assay mixture contained: (a) for Pol I, 50 mM Tris/HCl pH 8.8, 1 mM dithiothreitol, 10 mM KCl, 2 mM $MgCl_2$, 0.4 mg·mL^{-1} BSA, 20 µM [^{3}H]dTTP (1.5 Ci·mmol^{-1}), 0.42 µM of poly(dA)$_{265}$/oligo(dT)$_{17}$ (10 : 1), 200 µM (each) dATP, dGTP, dCTP and dTTP and 1 µL of the enzyme fraction; (b) for Pol II, 20 mM potassium phosphate (pH 6.5), 2 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 20 µM [^{3}H]dTTP (1.5 Ci·mmol^{-1}), 0.42 µM of poly(dA)$_{265}$/oligo(dT)$_{17}$ (10 : 1), 200 µM (each) dATP, dGTP, dCTP and dTTP and 1 µL of the enzyme fraction. All reactions were incubated at 65 °C for 30 min. The amount of radioactivity incorporated into DNA strands was measured in a scintillation counter. One unit is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of dTMP per min at 65 °C into polydA/oligodT.

Exonuclease activity assay

Associated exonuclease activities were assayed using previously described methods [12,21]. For the 3′→5′ exonuclease assay, pBluescript II SK(−) [pBS SK(−)] was digested by *Eco*RI, purified and the resultant linear fragment was labeled at the 3′ end by use of the Klenow enzyme in the presence of [^{3}H]dTTP. The 5′→3′ exonuclease activity was measured using a 446-bp *Eco*RI pBS SK(−) fragment labeled at the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase. Two hundred and fifty nanogams of 3′-labeled DNA and 8 ng of 5′-labeled DNA were used for 3′→5′ exonuclease and 5′→3′ exonuclease assays, respectively. The 20-µL reaction mixture contained for (a) Pol I: 50 mM Tris/HCl pH 8.8, 1 mM dithiothreitol, 60 mM KCl, 2 mM $MgCl_2$, 0.4 mg·mL^{-1} BSA, 3′ or 5′-labeled DNA and 0.3 U of purified DNA polymerase; (b) Pol II: 20 mM potassium phosphate (pH 6.5), 2 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 250 ng 3′- or 5′-labeled DNA and 0.3 U of purified DNA polymerase. The mixtures were incubated 65 °C for 90 min. The release of acid-soluble nucleotides was scored by adding 60 µL of trichloroacetic acid (10%) and 3 mg·mL^{-1} BSA to the 20-µL reaction. After incubating for 10 min on ice, the samples were centrifuged at 12 000 ***g*** for 5 min. The acid-soluble radioactivity in 60 µL of supernatant was quantified using Pico-Fluor 15 (Packard) scintillation fluid in a scintillation counter.

Optima pH, thermal stability, substrate specificities

The optimum pHs for the two DNA polymerases were determined by carrying out the standard assay at 65 °C, using (a) for Pol I: Tris/HCl (50 mM) and glycine/NaOH (50 mM) buffers for the pH range 7–10 and 8–11, respectively; (b) for Pol II: potassium phosphate (20 mM) and Tris/HCl (50 mM) buffers for the pH range 6–8 and 7–9.5, respectively. Thermostability was determined by use of purified enzymes (0.8 mg·mL^{-1} in buffer C for Pol I and 0.5 mg·mL^{-1} in buffer B for Pol II) and incubated at 70, 80, 90 and 100 °C for 2 h. Residual activity was determined by standard procedures at 65 °C. To compare the substrate specificities, of the two *P. abyssi* DNA polymerases, the standard assay was run with the following substrates: poly(dA)$_{265}$/oligo(dT)$_{17}$ (10 : 1) (0.42 µM, nucleotides concentration), primed M13 circular ssDNA (M13 DNA) (2.1 µM, nucleotide concentration) and activated calf thymus DNA (13 µg·mL^{-1}).

RESULTS AND DISCUSSION

Cloning of the two DNA polymerases genes from *P. abyssi*

The *polI* gene, encoding a family B DNA polymerase, and the *polB* and *polC* genes, encoding a heterodimeric family D DNA polymerase [8] were identified among the 1765 ORFs in the complete genome sequence of *P. abyssi* (available at http://www.genoscope.fr/Pab/). The 2313-bp *polI* gene is located at positions 1 695 183–1 697 495 on the *P. abyssi* chromosome. Its predicted product is a protein of 771 amino-acids, with a theoretical molecular mass of 84.8 kDa. In *P. abyssi*, *polB* (1857 bp) and *polC* (4365 bp) of the family D DNA polymerase, are arranged in tandem and located at positions 115 179–121 402 on the *P. abyssi* genome. The *PolC* gene, encoding the large subunit of Pol II, was shown to contain an intein which is an intervening sequence spliced out as a protein and not as a mRNA [22]. After the production of the precursor protein, the intein is excised from the protein. To prevent a possible toxic effect of the intein in *E. coli*, the

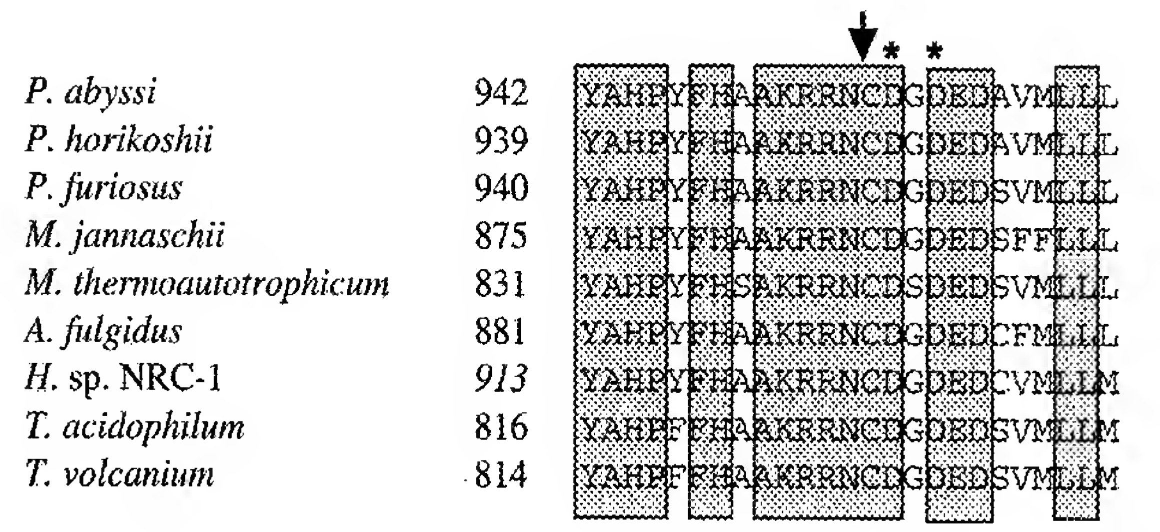

Fig. 2. Comparison of the conserved amino-acid sequence region in the active site of the family D DNA polymerases. The two proposed catalytic residues [27] are marked by an asterisk. The arrow indicates the position of the mini-intein insertion in *P. abyssi, P. horikoshii and Halobacterium* sp. NRC-1. Conserved identical residues are highlighted in grey. Sequences were deduced from the following accession numbers (SWISSPROT amino-acids sequence database): *P. horikoshii* (Q57861), *P. abyssi* (Q9V2F4), *P. furiosus* (P91409), *M. jannaschii* (*Q59024*), *M. thermoautotrophicum* (*O27579*), *A. fulgidus* (*O28552*), *H.* sp. NRC-1 (Q9HMX2), *T. acidophilum* (*Q9HM33*), *T. volcanium* (complete genome NC_002689, accession NP_110554).

555-bp gene encoding intein was deleted, resulting in *polC2*. Three of the nine family D DNA polymerase sequences from the database were shown to be inserted by a mini-intein: *P. abyssi* (185 amino-acids length), *P. horikoshii* (166 amino-acids in length) and *Halobacterium* sp. NRC-1 (195 amino-acids length) [27]. Interestingly, in these organisms, the mini-intein is inserted in the most conserved region of the family D DNA polymerase in close vicinity of the two catalytic aspartic residues (Fig. 2). Thus, the deduced product of *polB* and *polC2* genes are proteins of 619 and 1270 amino-and acids, with theoretical molecular masses of 68.1 and 139.7 kDa, respectively. The organization of the genes encoding the DNA polymerase D components is interesting (Fig. 1). The two genes responsible are arranged in tandem in the *P. abyssi* genome and the regions flanking *polB* and *polC* contain genes that encode proteins that are essential for DNA replication. These include a homologue of the initiator protein Orc1/Cdc6, the two subunits of the polymerase accessory complex replication factor C and the identified origin of replication (*oriC*) [23,24]. The organization of these genes is similar in the genus *Pyrococcus* (*P. furiosus*, *P. woesei and P. horikoshii*) [8]. Although the family D of DNA polymerases has only been detected in the euryarchaeal subdomain of archaea, the tandem organization of the genes encoding the two subunits of the heterodimeric polymerase seems to be specific to the Thermococcales: *Pyrococcus* (*P. furiosus, P. woesei* [8] and *P. abyssi*, present work) and *Thermococcus* (*T. aggregans*, K. Böhlke, Institute of Technical Microbiology, Hamburg, Germany, personal communication). The significance of these differences in gene arrangement is not known. However, we expect that the organization as an operon could be advantageous to regulate the level of the two DNA polymerase subunits in the cells.

Comparison of the amino-acid sequences of Pol I and Pol II with other DNA polymerases

The sequences of the two *P. abyssi* DNA polymerases were aligned with those of archaeal DNA polymerases available in public databases. The amino-acid sequence of Pol I contains the six conserved motifs shared by the family B DNA polymerases and the three motifs for 3′→5′ exonuclease activity [13]. The Pol I sequence from *P. abyssi* showed highest identities to the euryarchaeal DNA polymerases from the family B than to the crenarchaeal family B DNA polymerases (Table 1). These results are consistent with the report on the phylogenetic relationships of the archaeal family B DNA polymerases [13]. Comparison of the deduced primary sequences of *polB* and *polC* with the sequences of enzymes present in public databases indicated that they are highly similar to representatives of the family D DNA polymerases, which only contains heterodimeric DNA polymerases from euryarchaea. So far, only nine family D DNA polymerase sequences have been deposited in the genetic database. The two subunits of the heterodimeric polymerases are highly conserved within the representatives of the DNA polymerases family D, suggesting that these proteins play an important role in the organisms. Comparison of *P. abyssi* Pol II to members of family D revealed identities of between 79% (*P. horikoshii*) and 28% (*M. jannashii*) for the small subunits and between 91% (*P. horikoshii*) and 44% (*Thermoplasma acidophilum*) for the large subunit. In addition, as previously reported in other euryarchaea [25], the small subunit of *P. abyssi* has significant similarities to the small subunit of the eukaryotic pol δ and, to a lesser extent, to the small subunit of the eukaryotic pol α and pol ε. Furthermore, although the large subunit of the family D DNA polymerases exhibits no similarity to the catalytic domain of any known DNA polymerases from the database, amino-acid sequence analysis showed that it contains conserved motifs, including invariant carboxylates found in the palm domain of the polymerase superfamily [8,26]. However, site directed mutagenesis of this residue showed that they are not involved in the DNA polymerization activity of *P. horikoshii* family D DNA polymerase [27]. In the same study, two aspartate residues, located in a highly conserved region of the large subunit were proposed, based on site directed

Table 1. Comparison of the amino-acid sequences of archaeal family B DNA polymerases. Identity values were calculated using the GAP program (GCG Wisconsin Package) in pairwise comparison. Nomenclature of the polymerase (B1 and B3) is that of Edgell *et al.* [13].

	Amino-acid identity (%)										
Organism	*Pab*	*Pho*	*Pfu*	*Tli*	*Mja*	*Afu*	*Poc* B3	*Sso* B3	*Poc* B1	*Sso* B1	*Spo*
Euryarchaea											
P. abyssi	100	92	84	76	47	37	35	25	26	26	29
P. horikoshii		100	82	75	46	37	34	24	26	25	29
P. furiosus			100	74	47	37	35	25	25	27	28
T. litorallis				100	47	38	33	24	25	26	28
M. jannashii					100	36	32	24	23	26	25
A. fulgidus						100	43	29	24	25	26
Crenarchaea											
P. occultum B3							100	33	26	25	27
S. solfataricus B3								100	18	18	20
P. occultum B1									100	50	19
S. solfataricus B1										100	19
Eukarya											
S. pombe PolD											100

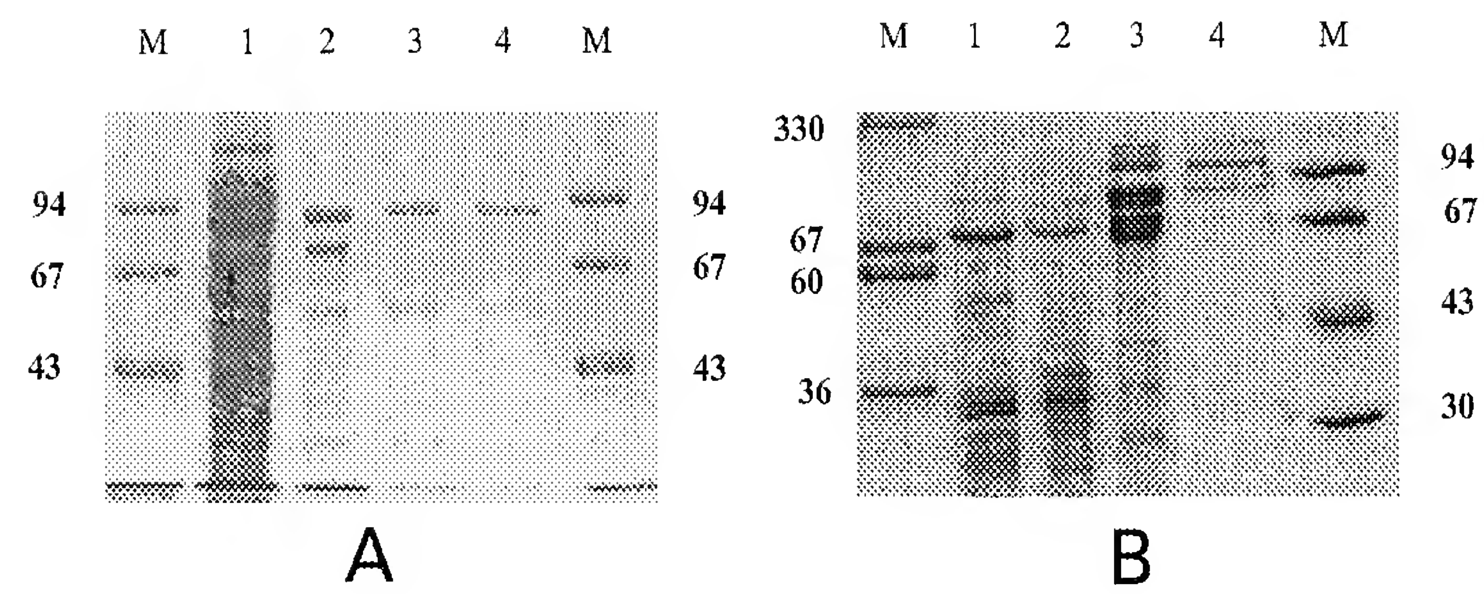

Fig. 3. SDS/PAGE of enzymatic fractions generated during the purification of Pol I (A) and Pol II (B). Lane 1, sonicated crude extract; lane 2, supernatant after heat treatment; lane 3, Resource-Q fraction, lane 4, heparin–Sepharose fraction. Lanes labeled 'M' contain size marker proteins (HMW and LMW calibration kits, Pharmacia). Molecular masses are indicated in the margins.

mutagenesis, to be the catalytic residues of the family D DNA polymerase (Fig. 2) [27].

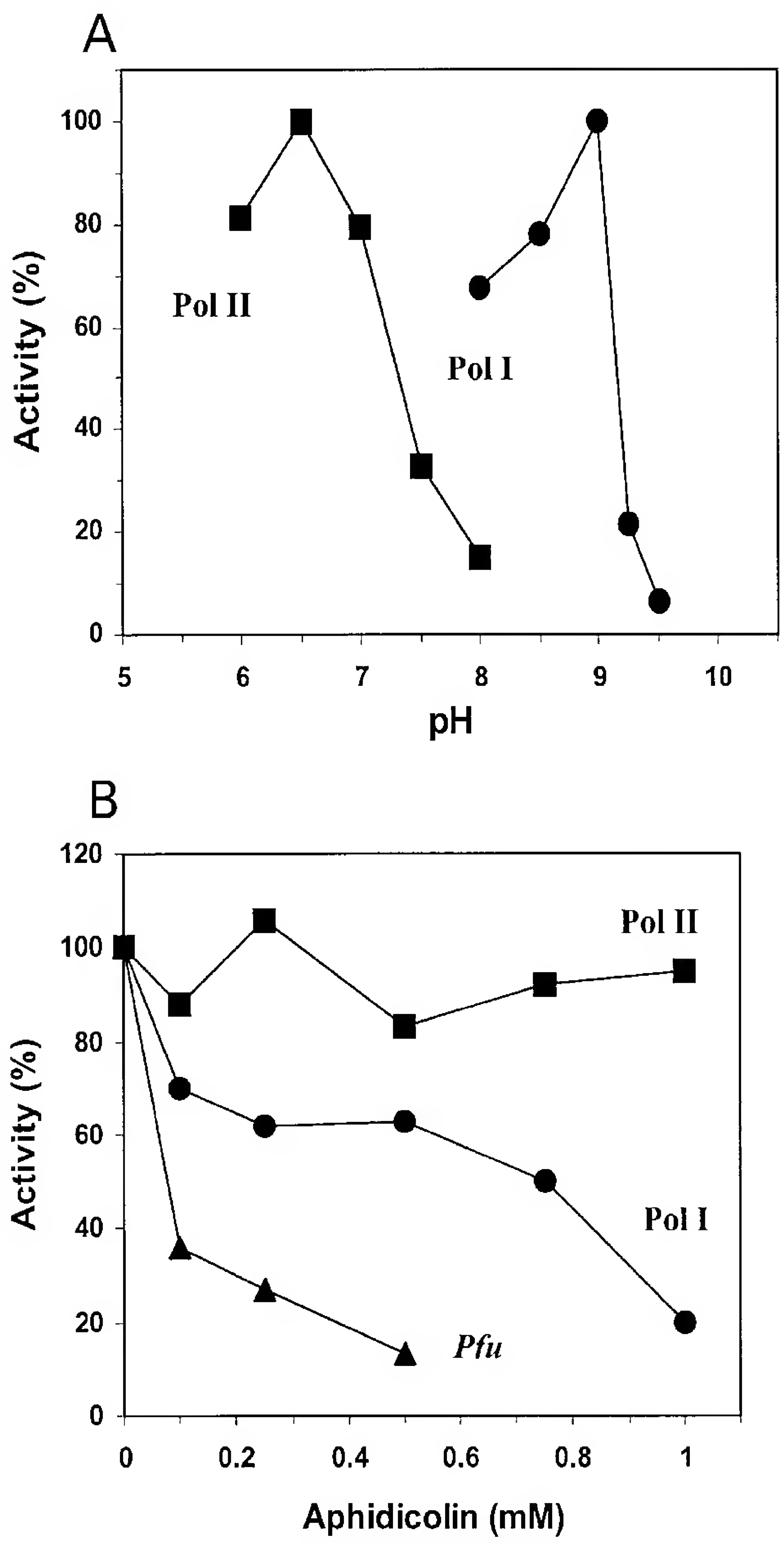

Fig. 4. Influence of pH (A) and aphidicolin (B) on the activities of *P. abyssi* Pol I and Pol II. (A) The standard assay was carried out at 65 °C for 30 min in 50 mM glycine-NaOH buffer for Pol I and in 20 mM potassium phosphate buffer for Pol II. One hundred percent activity corresponds to 80 $U \cdot mg^{-1}$ of protein for Pol I and 17 $U \cdot mg^{-1}$ of protein for Pol II. (B) The standard assay was carried out at 65 °C for 30 min with various concentrations of aphidicolin. One hundred percent activity corresponds to 74 $U \cdot mg^{-1}$ of protein for Pol I and 17 $U \cdot mg^{-1}$ of protein for Pol II. *Pfu*, *P. furiosus* DNA polymerase.

Expression and purification of *P. abyssi* Pol I and Pol II

To determine the properties of the two *P. abyssi* DNA polymerases, the *polI*, *polB* and *polC2* genes were cloned into pET-26b+, pARHS and pET-26b+, respectively, under the control of the T7 promoter. The resulting plasmids were checked by DNA sequence analysis. The two DNA polymerases were produced in recombinant form in *E. coli* HMS174(DE3)pLysS cells after IPTG induction. Pol I and Pol II were purified to near-homogeneity by a three-step procedure: (a) incubation at a high temperature that denatures most of the *E. coli* proteins; (c) anion exchange; and (c) Hi-Trap-heparin column chromatography (data not shown). SDS/PAGE analysis of the homogeneous proteins revealed a single band of ≈90-kDa for Pol I (Fig. 3A) corresponding to the calculated molecular mass of the *polI* product and three bands of ≈140, 110 and 90 kDa for Pol II (Fig. 3B). The two subunits of the heterodimeric Pol II were also expressed separately in *E. coli* HMS174(DE3), pLysS (data not shown). The 90-kDa band corresponded to the small subunit of Pol II, although its predicted molecular mass was 68.1 kDa. Similar results were obtained for the small subunit of the heterodimeric DNA polymerase of *P. furiosus* [28] and *P. horikoshii* [27]. The N-terminal sequences of the 140- and 110-kDa bands were analyzed after electroblotting from a denaturating gel to a poly(vinylidene difluoride) membrane. These sequences were found to be identical to the N-terminal sequence of the large subunit of Pol II. The 140-kDa band is the same size as the calculated molecular mass of *polC2* product and the 110-kDa band probably corresponds to a degradation product of the 140-kDa large subunit of Pol II. So far, only three family D DNA polymerases have been characterized [27–29]. The different studies showed that purification of the *P. furiosus* and *P. horikoshii* family D DNA polymerases resulted in the rapid degradation of the small subunit for *P. furiosus* and the large subunit *P. horikoshii* during the different steps [27,28]. This suggests that family D DNA polymerases are highly unstable during the purification procedure. *P. abyssi* Pol II migrated as a 230-kDa band in native PAGE, which is equal to the sum of the molecular masses of the deduced amino-acid sequences of the two subunits. This suggests that, as for *P. furiosus*, the active DNA polymerase complex is a heterodimer made up of a 1 : 1 ratio of the two Pol II subunits [8,28]. On the other hand, based on gel filtration results, the structure of *P. horikoshii* recombinant family D DNA polymerase was shown to be different; the results suggested a

Table 2. Activities of DNA polymerases on various template primers. *Pfu*, *P. furiosus* DNA polymerase. *Taq*, *Taq* DNA polymerase.

Substrate	Relative activity Pol I	Pol II	*Pfu*	*Taq*
Calf thymus-activated DNA	1.00	1.00	1.00	1.00
M13 ssDNA 30-mer primer	2.48	2.85	0.79	2.83
Poly(dA)-oligo(dT) (10 : 1)	7.45	1.92	3.13	2.18

heterotetrameric structure containing two large and two small subunits in one molecule of 421-kDa [27]. Moreover, when either of the two Pol II subunits were individually expressed in *E. coli,* no polymerizing activity could be detected in sonicated or heat-treated crude extracts. However, when the two extracts were mixed together, the activity was restored.

Biochemical properties of *P. abyssi* Pol I and Pol II

We compared substrate specificity, thermostability, pH dependency, salt dependency and sensitivity to aphidicolin of the two DNA polymerases. The pH dependencies of Pol I and Pol II were clearly distinct. Pol I was more active at pH 8.5–9 in a glycine/NaOH buffer, whereas Pol II showed optimal activity in a potassium phosphate buffer at pH 6.5 (Fig. 4A). Tris/HCl buffer seemed to extend the range of activity of both Pol I and Pol II; in 50 mM Tris/HCl buffer, both Pol I and Pol II retained more than 80% of their optimal activity between pH range 7–10 and 7–8.5, respectively (data not shown). We compared the optimal concentrations of $MgCl_2$ and KCl required for the incorporation reaction using activated DNA at the optimal pH. Pol I had an optimum $MgCl_2$ concentration of 3 mM and Pol II had an optimum range of 15–20 mM (Fig. 5A). The optimal concentrations of KCl were 50–80 mM for Pol I and 40 mM for Pol II (Fig. 5B).

The optimal temperature for polymerase activity could not be measured because activated DNA was not stable above 75 °C. The thermostability of Pol I and Pol II was tested at different temperatures: 70, 80, 90 and 100 °C (Fig. 5C). Pol I was found to be more thermotolerant than Pol II.

Table 3. Comparison of the exonuclease activities associated with DNA polymerases. One unit of DNA polymerizing activity is defined as described in Materials and methods. *Pfu*, *P. furiosus* DNA polymerase. *Taq*, *Taq* DNA polymerase. ND, non detectable.

DNA polymerase	DNA polymerizing activity (U)	Relative nucleolytic activity (%) 3′→5′ nucleolytic	5′→3′ nucleolytic
Pol I	0.5	42	ND
Pol II	0.5	100	ND
Pfu	0.5	35.7	ND
Taq	0.5	ND	100

The sensitivities of the two DNA polymerases to aphidicolin, an inhibitor of many eukaryal-type family B DNA polymerases, were compared. The activity of Pol I was inhibited by aphidicolin (Fig. 4B). However, Pol II was not inhibited at this range of drug concentration. Similar results were obtained with the already described family D DNA polymerases from *P. furiosus* [28] and *Methanococcus jannaschii* [29]. The substrate specificities of Pol I and Pol II are shown in Table 2. Pol I and Pol II utilized various types of DNA as template-primers. The highest activity of *P. abyssi* Pol II was measured with primed ssM13 DNA as a substrate and Pol I preferred the artificial substrate poly(dA)/oligo(dT). In contrast, activity on activated calf thymus DNA was ≈2.5-fold lower. These results support the notion that Pol I and Pol II may participate in DNA replication in *P. abyssi*. However, further experiments, specifically designed to study processivity of the two DNA polymerases, will give further insight into their potential cellular function as either a repair or a replicative enzyme. However, it is probable that some other associated proteins function together with these DNA polymerases for the *in vivo* synthesis of DNA.

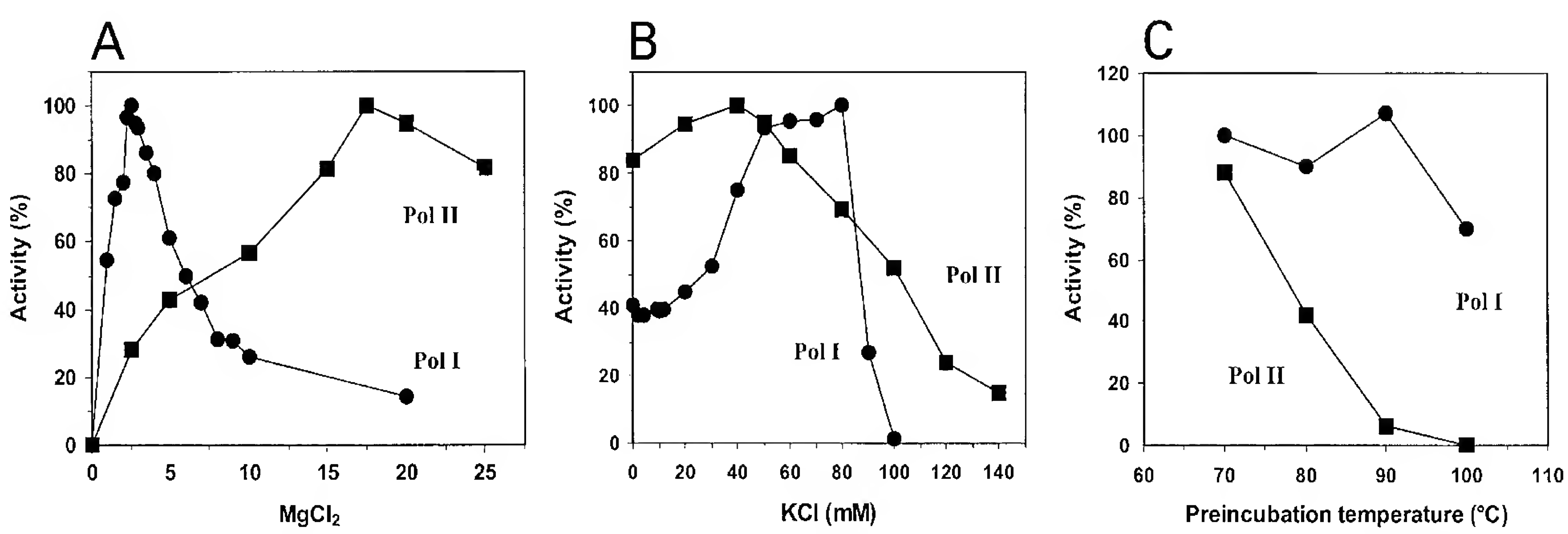

Fig. 5. Influence of magnesium ions (A), potassium ions (B) and thermostability (C) on the activities of *P. abyssi* Pol I and Pol II. (A) The standard assay was carried out at 65 °C for 30 min with various concentrations of $MgCl_2$. One hundred percent activity corresponds to 74 $U{\cdot}mg^{-1}$ of protein for Pol I and 35 $U{\cdot}mg^{-1}$ of protein for Pol II. (B) The standard assay was carried out at 65 °C for 30 min with various concentrations of KCl. One hundred percent activity corresponds to 150 $U{\cdot}mg^{-1}$ of protein for Pol I and 20 $U{\cdot}mg^{-1}$ of protein for Pol II. (C) The purified DNA polymerase was incubated at various temperatures for 2 h and its activity was measured at 65 °C for 30 min in the standard assay mixture. One hundred percent activity corresponds to 74 $U{\cdot}mg^{-1}$ of protein for Pol I and 17 $U{\cdot}mg^{-1}$ of protein for Pol II.

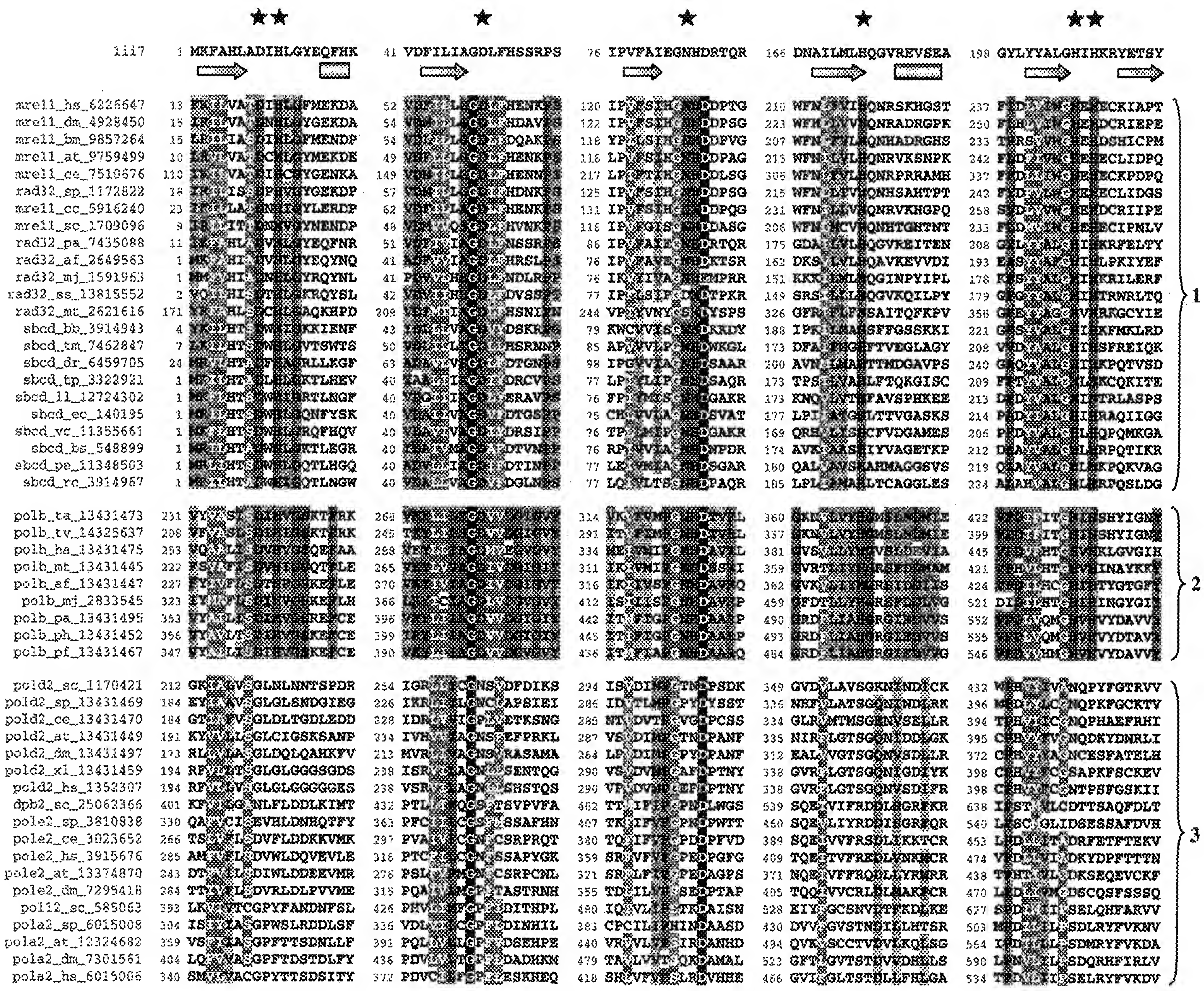

Fig. 6. The five phosphoesterase motifs in nucleases and DNA polymerases. Multiple alignments of the complete sequences have been constructed by DBCLUSTAL [35]. The first line represents the motifs in the Mre11 nuclease from *P. furiosus* for which the X-ray structure has been determined [33] (PDB:1ii7) with the deduced secondary structure (arrow for β strand, rectangle for α helix). The seven residues involved in metal coordination and catalysis are indicated by asterisks. Sequences are divided into three groups: (1) the Mre11/Rad32/Sbcd nuclease family; (2) the small subunit of the archaeal Pol II; (3) the small subunit of eukaryotic polymerases α, δ, ε. In groups 1 and 3, only sequences with < 70% identity are shown because of space limitations. Sequence names are composed of the protein abbreviation, the species abbreviation and the GI number of the original sequence. The numbers before the blocks indicate the position of the first residue in the protein sequence. The shading is based on a 90% consensus. Inverse shading indicates conservation across the three groups (inverse black shading shows residue conservation and inverse grey shading shows physicochemical properties conservation). Grey and light grey shading show residue and physicochemical property conservation within a group, respectively. Physicochemical groups considered are: small (P, G, S, T, A), aromatic (F, Y, W, H), hydrophobic (A, I, L, M, V, F, Y, W), positively charged (K, R, H) and polar residues (D, E, Q, N). The species abbreviations are as follows: hs, *Homo sapiens*; dm, *Drosophila melanogaster*; bm, *Bombyx mori*; at, *Arabidopsis thaliana*; ce, *Caenorhabditis elegans*; sp., *Schizosaccharomyces pombe*; cc, *Coprinus cinereus*; sc, *Saccharomyces cerevisiae*; pa, *P. abyssi*; af, *Archaeoglobus fulgidus*; mj, *Methanococcus jannaschii*; ss, *Sulfolobus solfataricus*; mt, *Methanobacterium thermoautotrophicum*; bb, *Borrelia burgdorferi*; tm, *Thermotoga maritima*; Dr, *Deinococcus radiodurans*; tp, *Treponema pallidum*; ll, *Lactococcus lactis*; ec, *Escherichia coli*; vc, *Vibrio cholerae*; bs, *Bacillus subtilis*; pe, *Pseudomonas aeruginosa*; rc, *Rhodobacter capsulatus*, ta, *Thermoplasma acidophilum*; tv, *Thermoplasma volcanium*; ha, *Halobacterium* sp.; ph, *P. horikoshii*; pf, *P. furiosus*: xl, *Xaenopus laevis*.

Associated exonuclease activities

Many DNA polymerases are known to have associated exonuclease activities. Therefore, we assayed the $5'\rightarrow3'$ and $3'\rightarrow5'$ exonuclease activities of the purified recombinant Pol I and Pol II (Table 3). No $5'\rightarrow3'$ exonuclease activity was detected for Pol I or Pol II. Conversely, both Pol I and Pol II were found to exhibit $3'\rightarrow5'$ exonuclease activity. Pol I and *Pfu* (Promega, used as a control) were shown to have similar exonuclease activity relative to their DNA polymerase activities, whereas the exonuclease activity of Pol II was 2.5-fold stronger (Table 3). Almost all archaeal family B DNA polymerases were known to have associated $3'\rightarrow5'$ exonuclease activity, which is responsible for correction of mismatched dNTPs. Three domains (Exo I, Exo II and Exo III) have been proposed to be essential for this activity [30]. These three domains were identified in the Pol I amino-acid sequence. In contrast, these three domains could not be found within the Pol II amino-acid sequence despite the associated $3'\rightarrow5'$ exonuclease activity. The three described family D DNA polymerases from *P. furiosus* [28], *M. jannaschii* [29] and *P. horikoshii* [27] were also shown to possess associated $3'\rightarrow5'$ exonuclease activity but no domain essential for this activity in this family has been

identified. The large subunit of *P. horikoshii* family D DNA polymerase has been investigated by site-directed mutagenesis of conserved aspartate or glutamate residues but none of the 28 reported mutations provoke the loss of exonuclease activity [27]. Sequence analysis has revealed that the small subunit of the euryarchaeal family D DNA polymerase, as well as the small subunit of eukaryotic pol α, δ and ε, belong to the large calcineurin-like phosphoesterase superfamily defined by five conserved motifs [31]. This superfamily consists of enzymes with a common di-metal active site [32] but with diverse functions such as protein phosphoserine phosphatases, nucleotidases, sphingomielin phosphodiesterases and nucleases. Aravind & Koonin [31] suggested that, in the small subunit of the family D DNA polymerase, the phosphoesterase domain may be involved in pyrophosphate hydrolysis. However, a crystal structure has recently deciphered a di-metal nuclease mechanism in one member of the phosphoesterase superfamily, the Mre11 nuclease of *P. furiosus* [33]. The seven reported residues involved in metal coordination and catalysis are conserved in the small subunit of the family D DNA polymerase but absent in the small subunit of eukaryotic pol α, δ and ε (Fig. 6). An appealing hypothesis is that the phosphoesterase domain of the small subunit is indeed responsible for the $3' \rightarrow 5'$ exonuclease activity of archaeal family D DNA polymerase through a similar divalent cation mechanism. In this case, the absence of the seven essential residues in the eukaryotic small subunits would reflect their loss of exonuclease activity. This is in agreement with experimental data: the $3' \rightarrow 5'$ exonuclease activity of pol δ and pol ε is provided by the large subunit and no enzymatic activity has been assigned so far to the small subunit [25]. If future site-directed mutagenesis studies confirm our hypothesis, both subunits of the archaeal family D DNA polymerase would be catalytic but with distinct activities: the small subunit carrying the $3' \rightarrow 5'$ exonuclease function and and the large subunit carrying the DNA polymerase function.

In vivo function of *P. abyssi* DNA polymerases

The study of DNA replication in general and DNA polymerases in particular in hyperthermophilic archaea is important and interesting in its own right and from an evolutionary point of view. Moreover, the revelation of that the replication machinery of archaea may have many of the basic components of the eukaryal replication machinery (origin recognition, helicase, clamp loader, elongation factor, DNA strand synthesis and ligation, primer synthesis, removal of primers) offers new opportunities for understanding the complexities of eukaryal systems. Studies on DNA replication in archaea have been initiated recently and a few proteins involved in the process have been purified and characterized [24]. The complete *P. abyssi* genome sequence, which allowed the major proteins involved in DNA replication to be identified, combined with the identification of the replication origin [10], should provide important tools for future *in vivo* and *in vitro* studies on DNA replication, repair and recombination in this organism. This report represents the first step in the characterization of the DNA replication process in *P. abyssi*. *P. abyssi* eukaryal homologues of the replication factor C (RFC), proliferating cell nuclear antigen (PCNA) and replication protein A (RPA) have been expressed and characterized in the laboratory. Preliminary results clearly show that, as in eukarya and in the euryarchaeon *P. furiosus* [34], *P. abyssi* PCNA and RFC stimulate DNA synthesis by Pol I and Pol II in *P. abyssi* (J. P. Raffin, unpublished results). In addition, due to the properties of DNA polymerase family D, Cann & Ishino [8] and Shen *et al.* [27] proposed that the family represents the replicative DNA polymerase of euryarchaea. All these recent results suggest that studies on archaea will provide an insight into DNA replication and that further efforts will lead to exciting discoveries.

REFERENCES

1. Jonsson, Z.O. & Hubscher, U. (1997) Proliferating cell nuclear antigen: more than a clamp for DNA polymerases. *Bioessays* **19**, 967–975.
2. Woese, C.R., Kandler, O. & Wheelis, M.L. (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl Acad. Sci. USA* **87**, 4576–4579.
3. Kahler, M. & Antranikian, G. (2000) Cloning and characterization of a family B DNA polymerase from the hyperthermophilic crenarchaeon *Pyrobaculum islandicum*. *J. Bacteriol.* **182**, 655–663.
4. De Felice, M., Sensen, C.W., Charlebois, R.L., Rossi, M. & Pisani, F.M. (1999) Two DNA polymerase sliding clamps from the thermophilic archaeon *Sulfolobus solfataricus*. *J. Mol. Biol.* **291**, 47–57.
5. Bult, C.J., White, O., Olsen, G.J., Zhou, L., Fleischmann, R.D., Sutton, G.G., Blake, J.A., FitzGerald, L.M., Clayton, R.A., Gocayne, J.D. et al. (1996) Complete genome sequence of the methanogenic Archaeon, *Methanococcus jannaschii*. *Science* **273**, 1058–1073.
6. Klenk, H.P., Clayton, R.A., Tomb, J.F., White, O., Nelson, K.E., Ketchum, K.A., Dodson, R.J., Gwinn, M., Hickey, E.K., Peterson, J.D. et al. (1997) The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. *Nature* **390**, 364–370.
7. Kawarabayasi, Y., Sawada, M., Horikawa, H., Haikawa, Y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kogusi, H., Hosoyama, A. et al. (1998) Complete sequence of the gene organization of the genome of a hyper-thermophilic archaebacterium, *Pyrococcus horikoshii* OT3. *DNA Res.* **5**, 147–155.
8. Cann, I.K.O. & Ishino, Y. (1999) Archaeal DNA replication: Identifying the pieces to solve a puzzle. *Genetics* **152**, 1249–1267.
9. Kawarabayasi, Y., Hino, Y., Horikawa, H., Yamazaki, S., Haikawa, Y., Jin-no, K., Takahashi, M., Sekine, M., Baba, S., Ankai, A. et al. (1999) Complete genome sequence of an aerobic hyper-thermophilic crenarchaeon, *Aeropyrum pernix* K1. *DNA Res.* **6**, 83–101.
10. Myllykallio, H. & Forterre, P. (2000) Mapping of a chromosome replication origin in an archaeon: response. *Trends Microbiol.* **8**, 537–539.
11. Cann, I.K.O., Ishino, S., Nomura, N., Sako, Y. & Ishino, Y. (1999) Two family B DNA polymerases from *Aeropyrum pernix*, an aerobic hyperthermophilic crenarchaeote. *J. Bacteriol.* **181**, 5984–5992.
12. Uemori, T., Ishino, Y., Doi, H. & Kato, I. (1995) The hyperthermophilic archaeon *Pyrodictium occultum* has two alpha-like DNA polymerases. *J. Bacteriol.* **177**, 2164–2177.
13. Edgell, D.R., Klenk, H.P. & Doolittle, W.F. (1997) Gene duplications in evolution of archaeal family B DNA polymerases. *J. Bacteriol.* **179**, 2632–2640.
14. Erauso, G., Reysenbach, A.L., Godfroy, A., Meunier, J.R., Crump, B., Partensky, F., Baross, J.A., Marteinsson, V., Barbier, G., Pace, N.R. et al. (1993) *Pyrococcus abyssi* sp. nov., a new

hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Arch. Microbiol.* **160**, 338–349.

15. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.
16. Ho, S.N., Pullen, J.K., Horton, R.M., Hunt, H.D. & Pease, L.R. (1990) DNA and protein engineering using the polymerase chain reaction: splicing by overlap extension. *DNA Prot. Eng. Tech.* **2**, 50–55.
17. de Moerlooze, L., Struman, I., Renard, A. & Martial, J.A. (1992) Stabilization of T7-promoter-based pARHS expression vectors using the *parB* locus. *Gene* **21**, 91–93.
18. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
19. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
20. Henneke, G., Raffin, J.P., Ferrari, E., Jonsson, Z.O., Dietrich, J. & Hubscher, U. (2000) The PCNA from Thermococcus fumicolans functionally interacts with DNA polymerase delta. *Biochem. Biophys. Res. Commun.* **276**, 600–606.
21. Kong, H., Kucera, R.B. & Jack, W.E. (1993) Characterization of a DNA polymerase from the hyperthermophile Archae *Thermococcus litoralis*. Vent DNA polymerase, steady state kinetics, thermal stability, processivity, strand displacement, and exonuclease activities. *J. Biol. Chem.* **268**, 1965–1975.
22. Perler, F.B., Davis, E.O., Dean, G.E., Gimble, F.S., Jack, W.E., Neff, N., Noren, C.J., Thorner, J. & Belfort, M. (1994) Protein splicing: inteins and exteins – a definition of terms and recommended nomenclature. *Nucleic Acids Res.* **22**, 1125–1127.
23. Myllykallio, H., Lopez, P., Lopez Garcia, P., Heilig, R., Saurin, W., Zivanovic, Y., Philippe, H. & Forterre, P. (2000) Bacterial mode of replication with eukaryotic-like machinery in a hyperthermophilic archaeon. *Science* **288**, 2212–2215.
24. Kelman, Z. (2000) The replication origin of archaea is finally revealed. *Trends Biochem. Sci.* **25**, 521–523.
25. Makiniemi, M., Pospiech, H., Kilpelainen, S., Jokela, M., Vihinen, M. & Syvaoja, J.E. (1999) A novel family of DNA-polymerase-associated B subunits. *Trends Biochem. Sci.* **24**, 14–16.
26. Cann, I.K.O., Komori, K., Toh, H., Kanai, S. & Ishino, Y. (1998) A heterodimeric DNA polymerase: evidence that members of Euryarchaeota possess a distinct DNA polymerase. *Proc. Natl Acad. Sci. USA* **95**, 14250–14255.
27. Shen, Y., Musti, K., Hiramoto, M., Kikuchi, H., Kawarabayashi, Y. & Matsui, I. (2001) Invariant Asp1122 and Asp1124 are essential residues for polymerization catalysis of family D DNA polymerase from *Pyrococcus horikoshii*. *J. Biol. Chem.* **20**, 27376–27383.
28. Uemori, T., Sato, Y., Kato, I., Doi, H. & Ishino, Y. (1997) A novel DNA polymerase in the hyperthermophilic archaeon, *Pyrococcus furiosus*: gene cloning, expression and characterization. *Genes Cells* **2**, 499–512.
29. Ishino, Y., Komori, K., Cann, I.K.O. & Koga, Y. (1998) A novel DNA polymerase family found in Archaea. *J. Bacteriol.* **180**, 2232–2236.
30. Blanco, L., Bernad, A., Blasco, M.A. & Salas, M. (1991) A general structure for DNA-dependent DNA polymerases. *Gene* **100**, 27–38.
31. Aravind, L. & Koonin, E.V. (1998) Phosphodiestarase domains associated with DNA polymerases of diverse origins. *Nucleic Acids Res.* **26**, 3746–3752.
32. Villafranca, J.E., Kissinger, C.R. & Parge, H.E. (1996) Protein serine/threonine phosphatases. *Cur. Opin. Biotech.* **7**, 397–402.
33. Hopfner, K.P., Karcher, A., Kraig, L., Woo, T.T., Carney, J.P. & Tainer, J.A. (2001) Structural biochemistry an interaction architecture of the DNA double-stand break repair Mre11 nuclease and Rad50-ATPase. *Cell.* **105**, 473–485.
34. Cann, I.K.O., Ishino, S., Hayashi, I., Komori, K., Toh, H., Morikawa, K. & Ishino, Y. (1999) Functional interactions of a homolog of proliferating cell nuclear antigen with DNA polymerases in Archaea. *J. Bacteriol.* **181**, 6591–6599.
35. Thompson, J.D., Plewniak, F., Thierry, J. & Poch, O. (2000) DbClustal: rapid and reliable global multiple alignments of protein sequences detected by database searches. *Nucleic Acids Res.* **28**, 2919–2926.